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THE TEXAS JOURNAL OF SCIENCE

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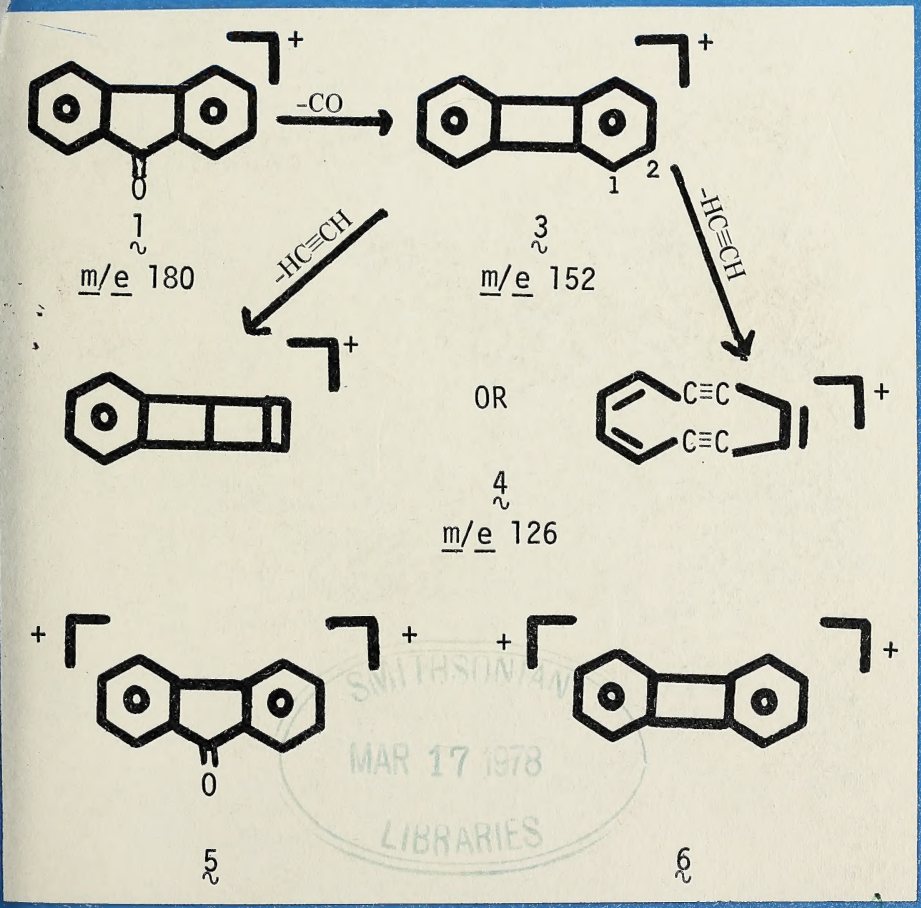

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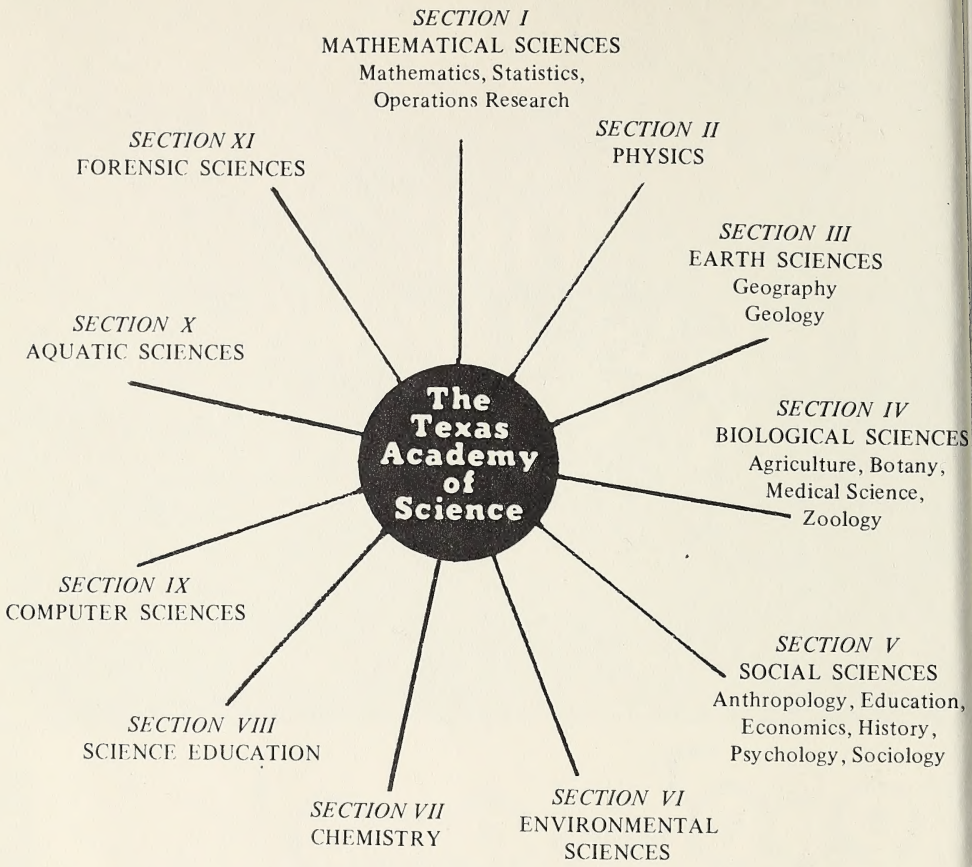

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PRESIDENT'S MESSAGE

THE TEXAS ACADEMY OF SCIENCE: RECENT PAST, PRESENT, FUTURE

James R. Underwood, Jr.
President

With the publication of this issue (Vol. 30, No. 1), the *Texas Journal of Science* is back on schedule. All of us owe Managing Editor Mike Carlo and Manuscript Editor Roland Vela a hearty vote of thanks. Dedicated effort was not in itself adequate to return the journal to its original schedule; this effort had to be supported by several changes in journal operations:

1. Institution of mandatory page charges.
2. Institution of pre-publication payment of page and reprint charges.
3. Use of the IBM composer for efficient processing of manuscripts.

The most critical ingredient during this period of uncertainty, however, has been the patience shown by academy members and by subscribing libraries. For that, we all are grateful.

The indebtedness of the Academy, which only two years ago totaled approximately $16,000, has been eliminated by:

1. Monthly meetings during 1976 of the Board of Directors to budget one month at a time, the academy's fiscal operations. During 1977 the Board of Directors met quarterly to review the financial position of the academy and to prepare and approve quarterly budgets.
2. Increasing membership dues.
3. The receipt of gifts and donations, including one anonymous and very generous gift of stock worth in excess of $13,000.
4. Placing the *Journal* on a sound financial basis.
5. Strict economizing in the operation of the office of the Secretary-Treasurer at Sam Houston State University and the *Journal* office at Angelo State University.
6. Applying for and receiving waiver of penalties incurred for earlier unintentional failure to file reports with the Internal Revenue Service and the Texas Employment Commission.
7. Continuing professionally successful and financially sound annual meetings.
8. Maintaining accurate books and records. An audit was held of the academy's records and its books at the close of 1976 and presented to the annual meeting at Baylor University in March 1977. The audit for 1977 is in progress and will be presented at the 81st annual meeting at Texas Tech University, March 9-11.

As a result of the improved fiscal status, attention now has been turned to the program of the academy, and the following developments are significant:

1. The Board of Development (not to be confused with the Board of Directors) has met often throughout 1976 and 1977 to offer guidance in fiscal affairs and program improvement and expansion.
2. Initial steps have been taken to establish a Texas Academy of Science Foundation to seek and manage endowment funds. At the 1978 annual business meeting at Texas Tech University, a constitutional amendment establishing the foundation will be presented for consideration.
3. When the Texas Academy of Science Foundation becomes a reality, the Academy will need, and the Foundation can support, an Executive Director and a permanent office.
4. The Collegiate and Junior Academies have been restructured and strengthened and continue to be active and vital parts of the total program of the Academy.
5. The Membership Committee of the Academy now has a broad regional structure and is moving rapidly to increase membership. The special emphasis is on regaining former Academy members and on recruiting in the Junior and Senior High Schools, Community Colleges, and in those Universities and Colleges where Academy membership is weak. Of special interest are those potential members in industry and government.
6. The Academy has continued to grant research awards, through AAAS, to outstanding High School students.
7. The newsletter of the Texas Academy of Science has been reinstituted and is serving as an efficient and valuable means of communication.
8. The Academy has been restructured and strengthened and the new constitution (1977) reflects these changes.
9. New sections have been inaugurated in Computer Sciences, Aquatic Sciences, and Forensic Sciences.
10. Through its Conservation Committee, the Academy has supported the Texas Committee on Natural Resources which is playing a major role in the establishment of the natural systems areas of the State of Texas.
11. The Science Education Committee has done outstanding work in the coordination of science education by industry in the State of Texas.
12. Beginning with the 1979 annual meeting at The University of Texas at Arlington, the Academy will publish reprints of papers presented at the annual meeting.
13. The first steps have been taken to establish *regional* TAS activities.

The present financial condition and program of the Academy, which should be a source of considerable pride to Academy members and friends, has been achieved through the dedicated efforts, patience, generosity, and perseverance of many members and officers, libraries who subscribe to the *Journal*, and state and federal officials. The attention of all of us now can be focused on improving our service to the physical, biological, and social scientific communities of the State of Texas through expansion of present programs and institution of innovative new ones. Your input is earnestly sought in a variety of ways:

1. Participation in your Section affairs.
2. Participation in the annual meeting of the Academy, including especially the business meetings of your Section and of the Academy.
3. Recruitment of members in your geographical area and within your TAS section.
4. Support of Junior and Collegiate Academies.
5. Preparation of papers for publication in the *Journal* and for presentation at annual meetings.
6. Review of manuscripts submitted to the *Journal*.
7. Support of conservation and science education in the State of Texas.
8. Forwarding suggestions for improvement in all phases of Academy operations and activities.

In all walks of life we owe much to the dedicated efforts of people who have preceeded us. In its more than 80 years, the Academy has enjoyed the support literally of thousands of people, and it has a rich heritage and strong traditions. What we, in this challenging time, make of these is limited only by our energy and our imagination. Let us be about the business of applying our talents to the enrichment of the Texas Academy of Science and of the State of Texas.

CALL FOR TEXAS ACADEMY OF SCIENCE HISTORICAL MATTER

The Texas Academy of Science is in the process of establishing an official repository for all of its past records, official papers, correspondence, and publications. This repository will serve the dual purpose of housing all the records of the Academy in one place and of providing the information necessary to prepare a written history of the Academy. Anyone having documents which pertain to the functions of the Academy in the past are urged to send them to Ms. Ethel W. McLemore, 11625 Wander Lane, Dallas, Texas 75230. All past officers and Board Members are especially urged to send the information they acquired while in office to Ms. McLemore. Please remember that the Academy needs your help in this matter and will be appreciative of any and all help that you can give.

TEXAS ACADEMY OF SCIENCE

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PALYNOLOGY: A USEFUL METHOD FOR DETERMINING PALEO-ENVIRONMENT

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ABSTRACT

Although a fairly new scientific discipline, palynology is now becoming one of the most common methods used in the reconstruction of paleoenvironments. Through the recovery and analysis of fossil pollen grains palynologists are able to reconstruct past vegetations, and through inference, suggest possible paleoclimatic conditions for the past.

INTRODUCTION

Palynology may not be a household word but what it represents is well known to many people. The term palynology was originally introduced by Hyde and Williams (1944) to represent all aspects of the study of pollen and spores, since at that time there was no unified term to refer to the discipline as a whole. Today many of us are well aware of pollen and are especially familiar with its allergenic effects. However, allergenic research is only one of many areas of study in the discipline of palynology.

There are many fields of palynological research, but this paper will devote its discussion to only one of them known as "paleopalynology" or "pollen analysis". Pollen analysis is a subfield within the discipline of palynology that is directly concerned with the reconstruction of past environments through the recovery, identification and statistical analysis of preserved fossil pollen. This is possible because the outer wall of pollen grains and spores contain a very durable compound called sporopollenin which is composed of an oxidative co-polymer of carotenoid and carotenoid esters (Brooks, 1971). Because of its great durability and resistance to destruction by most types of weathering agents, pollen grains and spores can be recovered from sediments of great antiquity. Fossil pollen has been recovered from sediments as old as the Pennsylvanian (more than 300 million years old). Fossil vascular plant spores have been recovered from Silurian deposits over 400 million years old, and fungal and algae spores have been found in sediments over a billion years old. Although fossil pollen and spores from any

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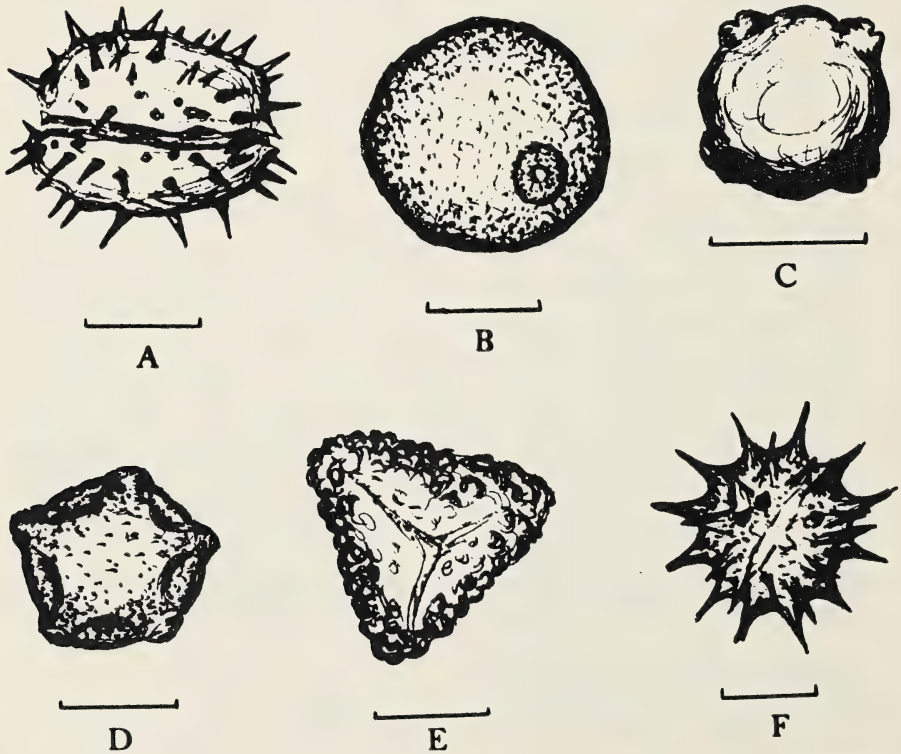
of these deposits can be used as a basis for attempting paleoenvironmental reconstructions, this discussion will be confined to the Quaternary Period since their records are of special interests to archeologists and many ecologists working with modern faunal and floral studies.

The term "pollen" is used to refer to the tiny male reproductive structures (male gametophytes) produced by seed-bearing plants. Spores, on the other hand, sometimes carry out the same designed function as pollen grains yet are produced by non seed-bearing plants such as the fungi, ferns, mosses and algae. The designed function of a pollen grain is to transport the male gametes, or sperms, to the female egg where fertilization can occur. For each pollen grain that achieves this function thousands fail and are instead deposited on the earth in the form of a pollen rain. Once deposited some of the pollen grains are oxidized or destroyed by microorganisms and/or chemical weathering. In some areas conditions are ideally suited for the preservation of pollen and in these regions many grains survive and become part of the fossil record.

Pollen Analysis

Pollen grains are tiny objects most of which are smaller than 100 μ and cannot be closely studied using only the unaided eye (Fig. 1). Ancient cultures such



(Figure 1)

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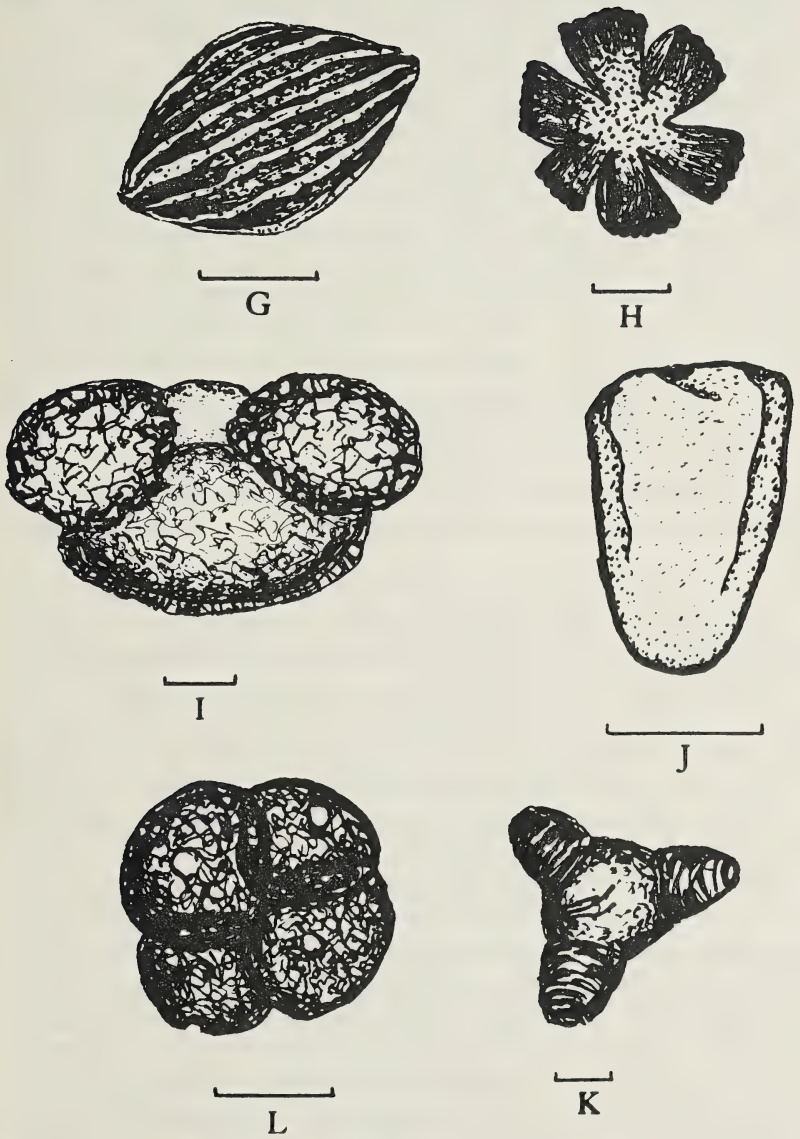

Figure 1. Selected pollen grain types. a) *Nuphar advena* (Pond lily) b) *Agropyron cristatum* (Quackgrass) c) *Myriophyllum spicatum* (Water milfoil) d) *Alnus rugosa* (Speckled alder) e) *Cyptogramma aristicoides* (Rock-brake) f) *Helianthus dalyi* (Sunflower) g) *Ephedra trifurca* (Joint-fir) h) *Monarda punctata* (Horsemint) i) *Abies balsamea* (Balsam fir) j) *Eleocharis obtusa* (Spikerush) k) *Gaura brachycarpa* (Gaura) l) *Typha latifolia* (Common cattail).

as the Assyrians recognized the importance of pollen in the production of date palms more than 2500 years ago yet the first critical study of pollen had to await the development of the compound microscope in the mid-1600's by Hooke. Shortly after the invention of the microscope two early scientists, Nehemiah Grew and Marcello Malphigi began studying aspects of plant physiology and noted that pollen grains from different plants had different morphological features. Although neither scientist fully understood the methods of plant fertilization, they both suspected that one role of the pollen grain might be in fertilization. Since the late 1600's many other scientists have expanded our knowledge concerning the role of pollen in fertilization, and pollen grain morphology.

In the late 1800's and early 1900's there was a great controversy among European scientists concerning the most accurate methods of reconstructing past environments. Some felt that this could best be done only through the identification and analysis of plant remains such as seeds, leaves, and fossilized woods. Others believed that it could best be accomplished through the study of geologic formations and interpreting the environmental conditions that must have occurred to produce those sediments. Neither of these approaches was entirely successful and unfortunately led to many misinterpretations which remained in the literature for many years.

The first step towards the discovery of the technique of pollen analysis occurred during the examination and identification of plant remains from Scandanavian peat bogs. A research assistant by the name of Gustav Lagerheim began counting the identifiable pollen grains which he encountered during a routine peat bog identification study (Faegri and Iversen, 1975). This represented the first actual pollen statistical count. Yet it was not until his associate Lennart von Post experimented with this technique that the real framework for the discipline of pollen analytical studies was formulated. In 1916 von Post presented the results of his initial pollen analysis at a convention of Scandanavian naturalists (von Post, 1916). In looking back at that report it is amazing how well he understood some of the important basic aspects of pollen analytical techniques; techniques which are still employed today. Since that initial study by von Post, palynologists in many areas of the world have continued to use this technique to attempt paleoenvironmental reconstructions and to use the resulting data to infer past climatic changes. Today the technique of pollen analysis is recognized as one of the most valuable tools used in formulating paleoenvironmental reconstructions. Although in some circumstances pollen analytical studies may be the only paleoenvironmental evidence available, it is still best conducted in circumstances where other types of evidence can also be used to help correlate the pollen data.

The Pollen Diagram

The validity of applying pollen analytical data to problems of paleoenvironmental reconstruction is based upon 5 premises: 1) most plants produce great

quantities of pollen or spores which are dispersed and carried by wind currents, water currents, or various types of animal vectors; 2) most pollen grains and spores which are produced by terrestial plants have a chemically stable outer wall (exine) which is highly resistant to deterioration; 3) pollen wall morphology is consistent within a given species and is generally different among unrelated plant taxa, thereby permitting the pollen of one genus or species to be recognized from types produced by other plants (Fig. 1); 4) most pollen and spore producing plants are restricted to the ecological habitats in which they can survive and are generally most abundant in their optimum environments, and 5) dispersed pollen which falls to the earth's surface in the form of a pollen rain generally provides a reasonably accurate image of the regional vegetation within a radius of approximately 30 mi.

Using proper laboratory extraction procedures it is possible to dissolve or remove the accumulated sediment surrounding the resistant pollen grains and then to concentrate the pollen for microscopic analysis. With the use of a microscope a statistically significant number of fossil pollen grains are then counted from each sample. The family, genus, and when possible the species, of each pollen grain is then recorded and tabulated. These pollen frequency totals from each individual sample are called a "pollen spectrum." When a series of these pollen spectra are presented graphically to form a continuous pollen diagram, the percentages of each pollen type within a given level are plotted against the other types (Fig. 2). Generally the time span or succession of strata is plotted on the vertical scale while comparative percentages of pollen at each level are plotted on the horizontal scale. The palynologist studies the occurrence, disappearance, and fluctuations in the percentages of fossil pollen types as recorded on the pollen diagram. Then he attempts to reconstruct the paleovegetational conditions that prevailed during the deposition of each stratum. Once the paleovegetational conditions have been reconstructed, the palynologist can, by inference, offer conclusions concerning the paleoclimatic conditions that may have existed during that period. It is important to remember that the palynologist is only able to reconstruct one aspect of the paleoenvironment: the paleovegetation. It is through the manipulation of that information that the palynologist is able to speculate on other aspects of the paleoenvironment.

Limitations and Problems of Pollen Analysis

Palynologists are not able to reconstruct a quantative distribution of precise vegetation types over a given geographical area during any point in time. At best the palynologist can only determine a generalized composition of past vegetation and must rely in part upon modern day analogs to help interpret the paleovegetational data. The palynologist's problems are complicated in this respect since the number of pollen grains that each plant species contributes to the local pollen rain depends upon 3 essential features: 1) the abundance and position of the plant in relation to the total vegetation cover, 2) the inherent pollen productivity capability of each plant species, and 3) the method of pollen dispersal.

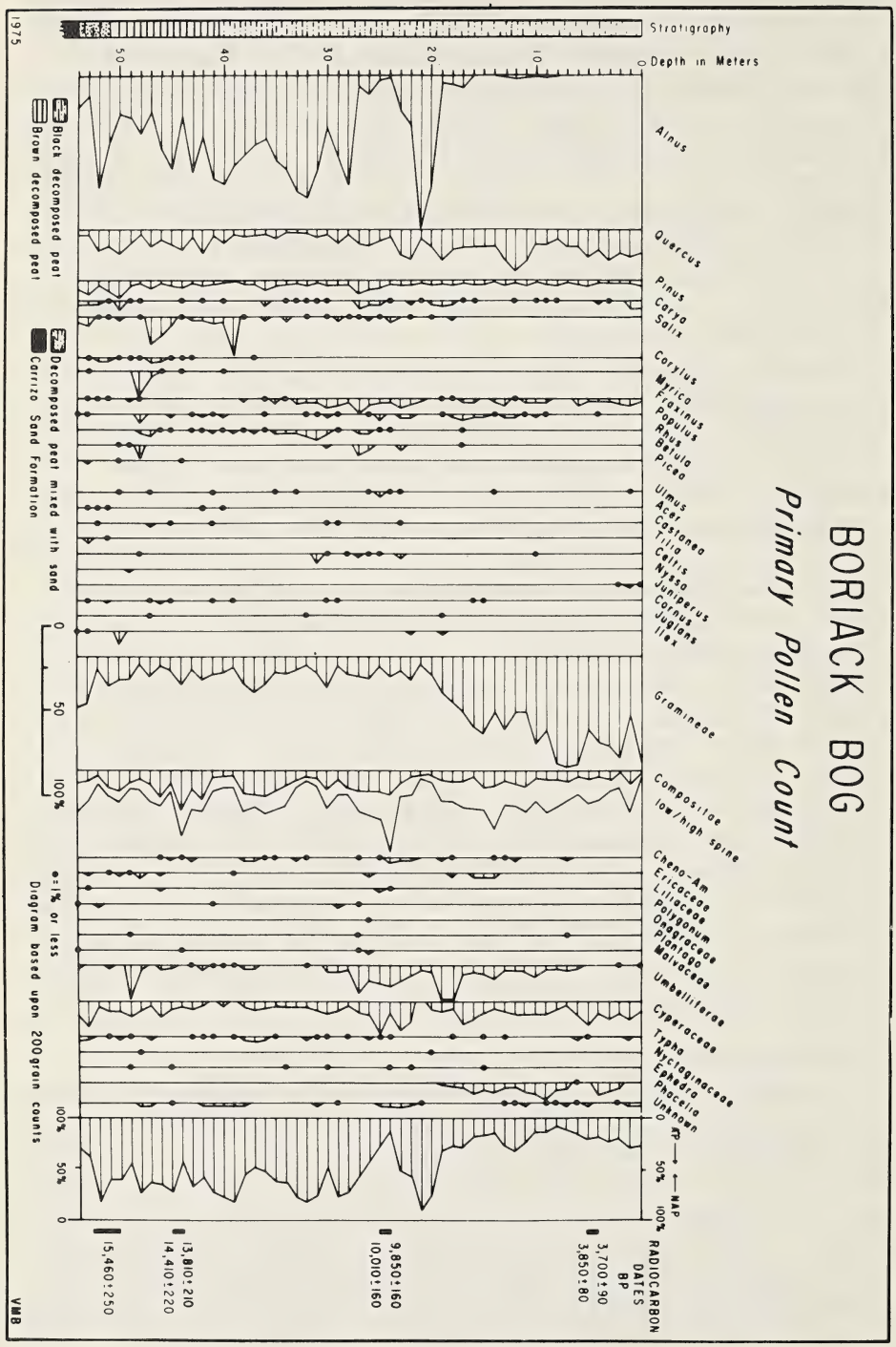

Figure 2. Boriack Bog pollen diagram of primary grain count.

There are a number of different methods by which plants disperse their pollen and each different method influences the percentage of pollen that will be deposited and can be recovered. Anemophilous (wind pollinated) plants produce the largest number of pollen grains, thus their pollen is best represented in the fossil record. Although the production of pollen varies among anemophilous plants most produce between 10,000 and 70,000 pollen grains/anther. Among some of the largest pollen producing anemophilous plants, such as the pines, it is not unusual for a single tree to produce several billion pollen grains. An idea of the enormity of pollen production among certain anemophilous plants is exemplified by the coniferous forests of middle and southern Sweden which are estimated to produce approximately 75,000 tons of pollen annually (Faegri and Iversen, 1975).

Zoophilous plants depend upon some type of animal (bat, bird, or insect) to transport the pollen from the anther of one flower to the stigma of another flower. The pollen grains from these plants are generally covered with layers of sticky oils (lipids) which hold them securely to the anther until they come in contact with an insect or animal. Insect pollinated plants produce far fewer numbers of pollen grains/anther (*ca.* 1,000 pollen grains) than do the anemophilous plants. Pollen from zoophilous plants is rarely found in the fossil record and when it does occur its presence is generally sporadic. Unless an anther or a whole flower is broken and falls to the ground or the pollen bearing transporter dies, the chance of zoophilous pollen becoming part of the fossil pollen record is slight.

There are other types of plants which produce pollen that is even less likely to become part of the fossil record. Autogamous plants have devised an extremely efficient method of self pollination. In this group of plants the pollen from each anther is used to directly pollinate the stigmatic surface of the same flower. Because of this high degree of efficiency little pollen production is needed. In most plants of this category pollen production is limited to less than 100 pollen grains per anther. It is only under unusual circumstances that any pollen from these types of plants is ever recovered in fossil pollen records. Hydrogamous plants produce pollen that is even less likely to be recovered from fossil deposits. Many of the plants which live completely submerged in water release their pollen and rely upon water currents to transport the pollen from the anther of one flower to the stigma of a neighboring flower. This method of transport is inefficient and therefore the plants produce great quantities of pollen. On the other hand, hydrogamous pollen almost always lacks the durable outer wall found in the wind and insect pollinated types. Without the durable outer wall, the pollen grain becomes very susceptible to destruction and therefore almost never remains preserved in fossil deposits or is unrecoverable using current pollen extraction procedures.

Another factor which must be taken into consideration by the palynologist is the degree of buoyancy of anemophilous pollen types. Certain wind pollinated grains, such as those produced by cattails, birch, and pine are well designed for

wind transportation and are very buoyant. As such they can travel many miles and in fact are often recovered many hundreds of miles or sometimes even thousands of miles from their point of origin. On the other hand, the larger and heavier wind pollinated types produced by fir trees and maize plants fall to the earth very quickly and are rarely distributed more than a few hundred yards from their source. Since these larger types seem to fall to earth more than 40 times faster than many of the lighter wind pollinated types (Leopold and Scott, 1957) they are not often distributed evenly within the regional pollen rain of an area. Factors such as the rate of pollen fall, pollen productivity, and method of dispersal must all be considered when trying to accurately reconstruct the past vegetation of any given area.

The above discussion of dispersal and productivity capabilities of plants indicates that pollen from anemophilous plants generally will be overrepresented in the local pollen rain whereas non-anemophilous pollen types usually will be either underrepresented or not represented at all. For this reason it is impossible to accurately or quantatively reconstruct the *exact* distribution of each plant type based solely upon the fossil pollen rain. However, the pollen spectrum from a given deposit will provide useful data concerning the overall types of vegetation in a given locale and a general idea of the plant associations that may have existed. From these data it is then possible to speculate what may have prevailed during some specific time period in the past.

Pollen Sampling

There are a large variety of accepted methods of collecting fossil pollen samples for analytical work and no effort will be made in this article to discuss each of these in detail. Instead, the discussion will be limited to some of the more widely used methods for collecting samples from Quaternary age deposits.

Peat bogs provide idea sampling locales because their pollen deposits generally reflect the composition of the neighboring upland vegetation and pollen preservation is generally excellent in the acidic, anaerobic environment of the bog (Tauber, 1965). In selecting a peat bog for sampling care must be taken not to collect samples from areas where redeposition and mixing of sediments have occurred in the past. Sampling locales that should be avoided include: 1) places where streams enter the bog; 2) small bogs less than 100 yds in diameter; 3) marginal areas along the edges of bogs and 4) bogs covered by dense vegetation.

Pollen sampling from bogs can best be achieved with the use of a Livingston Corer (Cushing and Wright, 1965). This sampling device consists of a large closed chamber that can be pushed down through peat or similar organic deposits. When the sampler reaches the desired depth the chamber can be opened from the surface and then forced downward until the empty chamber is filled. Once full, the chamber is then pulled to the surface and the solid peat core is extracted. Repeated sampling using this technique can result in the collection of a continuous core sample of peat extending from the surface to the bottom of the bog.

This method of sampling is especially used since each core segment can be quickly sealed in polyethylene wrapping and stored until examination of the deposit is possible. Furthermore, since a solid core is collected, the potential for contamination by modern pollen in the atmosphere is minimal. To insure a totally contamination free specimen the palynologist will generally extract material from the center of the core and use it for the actual analysis. A Hiller type auger can also be used for sampling bog deposits. It is a useful sampling tool but it is generally considered a less desirable technique since it does not produce a solid core of sediment.

A Livingston Core sampler can also be used in various types of lake deposits. These types of deposits are often more difficult to retrieve since it requires a floating platform from which to operate. In some circumstances sampling can best be carried out in winter by cutting a hole in the surface ice of a lake and coring through the opening.

Sediments deposited in locales other than bogs and lakes provide less desirable sampling locations because the fossil pollen yield is often much lower. This is generally true because pollen often remains unprotected on the surface of the ground and is subjected to many weathering agents which often destroy most of the pollen before it can be buried. Pollen analysis can be conducted from these types of deposits but they generally involve the collection and processing of much larger sediment samples and the use of lengthy and often complicated laboratory procedures.

Sampling techniques for terrestrial deposits are easy to conduct and do not require the use of expensive equipment. One of the more common techniques used for gathering samples from exposed river terraces, archaeological excavations, or open pits requires only the use of a shovel and trowel. The principle object is to secure samples free of atmospheric pollen contamination. To achieve this a 1-ft wide section of wall surface should be selected and then thoroughly cleaned from top to bottom by scraping away at least 6 in of surface sediment with a clean shovel. Once the cleaning is completed, sampling with a clean trowel can then begin. Often palynologists prefer to begin sampling with the lowest stratum and then continue up the column. By starting with the lowest stratum there is less probability that pollen and dirt from the upper strata will fall into the sampling area and thus contaminate the deposits below. Between samplings the trowel should be thoroughly cleaned using distilled water and clean paper towels to avoid contamination of the succeeding samples with dirt from earlier samples. The amount of sediment collected for each sample will vary with the composition of the site and the regional location. In soils where pollen preservation is poor, larger samples will be needed than in areas where pollen preservation is more nearly ideal. Under most circumstances samples consisting of at least 1 lb of sediment/sample is adequate for most laboratory extraction procedures.

Fossil Pollen Extraction

All laboratory techniques used in fossil pollen analysis consists of 2 basic steps. First, the removal of the unwanted matrices and the concentration of the fossil pollen. This is then followed by the actual pollen analysis. Hundreds of publications have appeared during the past 60 years containing statements on how to remove extraneous materials and concentrate fossil pollen in various types of sediments since each sediment type requires a slightly different laboratory procedure. However, most laboratory procedures include several basic techniques. Anhydrous carbonates of the calcite group are the most common type of carbonates found in sedimentary matrices likely to be examined for their pollen content. Carbonates such as these can be removed with hydrochloric acid or in some cases with glacial acetic acid. Silicates are generally removed using several techniques. The larger and coarser grain silicates can be initially separated using swirling or heavy density separation while the finer grain silicates can be removed using concentrated hydrous hydroflouric acid. The organic compounds that are generally found in sediments result from incomplete plant disintegration and consist of various types of resins, waxes, proteins, carbohydrates, derived carbohydrates (such as polyaccharides of cellulose and hemicellulose), and ligin in addition to sporopollenin from the outer wall of the fossil pollen or spores. One of the most effective methods of destroying the unwanted organic residues and not harming the fossil pollen and spores is to use the acetylation technique (Erdtman, 1960). In the acetylation procedure 9 parts of acetic anhydride is mixed with 1 part of concentrated H_2SO_4 and then heated. Most organic residues are destroyed in this solution by a process of hydrolysis and esterification leaving unchanged only the most durable materials such as the sporopollenin. Certain types of altered organic matter will not be completely dissolved using the acetylation method and thus must be removed by another process using an alkaline agent such as KOH. The aforementioned techniques are only a few of the many which are regularly incorporated into the laboratory phase of fossil pollen extraction.

Once the extraneous materials have been removed and the original sample has been reduced to a thin sludge containing mostly fossil pollen then it is ready to be mounted on slides for microscopic analysis. There are many types of mounting media and their useage will vary with the type and age of sediments and/or with the personal preferences of the individual palynologist. Two of the most widely used mounting media include glycerin jelly and silicon oil. The mounted slides are generally examined at magnifications between 400X and 1,000X and all fossil pollen grains are counted until a statistically significant number has been recorded. The number of pollen grains counted/sample will vary but under most circumstances palynologists feel that a minimum of at least 200 fossil pollen grains/sample should be counted (Barkley, 1934).

Pollen Preservation

Pollen analyses are costly since they involve many hours of laboratory time devoted to the extraction and analysis of the pollen material. Therefore it would

be extremely useful from the standpoint of expense and time if it were possible to predict in advance which sediments will contain fossil pollen and which will not. At present there are no known techniques which can tell a palynologist that type of information in advance. On the other hand, as research continues we now have a better understanding of conditions which tend to lessen the probability of recovering fossil pollen. This information is very useful in the sense that it can be used by potential researchers as an approximate guide to the recovery of the most ideal types of sediments which could be used for paleoenvironmental interpretations.

One of the first factors which tends to destroy pollen grains either before or during the preservation process is mechanical breakdown. Many pollen types tend to be very fragile and during their transportation on air currents they can become abraded when they strike each other or other objects. Once deposited on the ground they can become further abraded by the effects of water, wind, and soil movement.

In most sediments an even more important degradation factor is the chemical destruction of the pollen wall itself. Dimbleby (1957) has shown that soil pH appears to be an important factor in pollen preservation. He found that in soils with a pH greater than 6.0 the preservation of fossil pollen was not sufficient to warrant subsequent analysis. In studies which he conducted of soils from Guiana and regions of England he found that soils containing a highly acidic pH of 3.1 contained as many as 437,000 pollen grains/gram of dried sediment. On the other hand, he noted that with a less acidic soil pH of 5.5 fossil pollen recovery was only 2,400 pollen grains/gram dried sediment. Furthermore, his study demonstrated that in soils with a pH greater than 6.5 fossil pollen recovery was impossible. Dimbleby's study was an important contribution to the field of palynology yet later studies conducted by Martin (1963) showed that fossil pollen can be recovered from southwestern United States soils when the pH is greater than 6.5. In related studies of soils from west Texas it has also been noted that fossil pollen recovery is possible in highly alkaline soils with a pH as high as 8.9 (Bryant, 1969). However, it was found that when the soil pH is alkaline the fossil pollen grains tend to be highly corroded thereby making identification very difficult but not impossible.

Another factor which seems to play an important role in the preservation of fossil pollen in the oxidation-reduction potential (Eh) of sediments. Tschudy, (1969) has noted that sediments containing a low Eh indicate reducing environments and thus tend to be more favorable depositional environments for the preservation of fossil pollen than do sediments with a high Eh or an oxidizing environment.

The chemical composition of the pollen wall and the pollen wall structural morphology also play important roles in determining whether or not pollen grains will remain effectively preserved in various types of sediments. Havinga (1964) reported that he found the relationship between the percentage of sporopollenin

versus the percentage of cellulose in the pollen wall of a grain very much affects its susceptibility to destruction through oxidation. He found that grains having high percentages of sporopollenin in their walls tended to remain preserved longer than grains containing walls composed mostly of cellulose. Thus, as a rule the lower the percentage of sporopollenin in the pollen wall of a grain, the greater the susceptibility of that grain to destruction by oxidation. The actual morphological structure of the pollen wall also partly determines which pollen grains are most likely to remain preserved in certain sediments. Pollen grains having protruding structures from the sides such as bladders or spines (Fig. 1) can very often lose these appendages through a variety of degradation processes such as abrasion and/or chemical destruction. In some cases the actual appearance of a pollen grain can become so altered due to the loss of the appended structures that identification is impossible, or it causes severe weaknesses which hasten the destruction of the remainder of the grain through normal processes.

Certain types of biological agents such as fungi and bacteria have been shown to play a role in the degradation of pollen grains. Some of these microorganisms feed on the internal cytoplasm of pollen grains and thus aid in the destruction of the pollen grain by weakening the outer wall when they attempt to enter the pollen grain itself. Species of fungi such as the Phycomycetes were investigated by Goldstein (1960) and were found to be a positive factor in the destruction of certain pollen types. His data show that Phycomycetes tend to infect certain pollen types at a faster rate than others. For example, the pollen grains from coniferous trees tend to be more susceptible to destruction from the activity of Phycomycetes than do the pollen grains from certain types of deciduous trees and herbaceous plants. Elsik (1971) has also noted that bacterial degradation occurs in pollen. He found that some bacteria, especially the Actinomycetes, tend to degrade pollen walls in a definite pattern and that this activity can occur not only in fresh pollen still containing cytoplasm but can occur even after the pollen grain has become preserved. Elsik notes, in fact, that he believes that this process can still occur even after a grain has been preserved for thousands of years in various types of sediments.

Based upon these observations some palynologists have attempted to assess the pollen preservation potential of certain types of environments. In the early 1960's Sangster and Dale (1961, 1964) attempted to determine the rate of pollen preservation in 3 types of environments: a weakly acidic peat bog, a more acidic peat bog and a small alkaline pond. They found that the greatest amount of pollen destruction occurred in the pond deposit while lesser amounts of destruction occurred in both types of peat sediments. In a later study by Havinga (1971), mixtures of 19 different types of fresh pollen and spores were placed in porous nylon containers and were then buried in 5 different environments of deposition: a river clay soil, a sphagnum peat, a podsolized sand soil, a carex peat, and in a deposit of leaf mold. Based on his studies, Havinga concluded that the ratio of pollen destruction varied in each of the 5 sediment types. The

least destructive sediment to pollen was the sphagnum peat, and the most destructive was the deposit containing the leaf mold. The other 3 sediment types were intermediate and ranked from least to most destructive were: carex peat, podsolized sand, and river clay soil. More importantly, Havinga's study showed that there was differential pollen loss in many of these sediments types. For example, in the river clay soil and the leaf mold samples, the percentages of certain pollen types (alder, ash, poplar, willow and elm) were dramatically reduced while other pollen types in those same deposits showed only slight destruction. These data suggest that under certain circumstances the paleoenvironmental interpretation could easily be misinterpreted unless the palynologist was aware of the characteristics of the deposit.

Although palynologists have a general understanding of some of the mechanisms which destroy fossil pollen, we still do not fully understand how each of these agents may act separately or in combination with other agents in the ultimate destruction or preservation of fossil pollen. Another factor which may someday prove a useful guide to fossil pollen preservation in sediments is the % of organic content in the sediments. Thus far, preliminary experiments (Bryant, 1977a, 1977b) have shown that when soils contain less than 1% organic content, fossil pollen can not be recovered and analyzed. On the other hand, I have been able to recover sufficient fossil pollen for analysis from sediments containing an organic content as low as 1.6%. It is still too early to determine whether or not this technique will eventually be able to be used, with confidence, to determine the presence of preserved fossil pollen in soils. At present I am continuing to accumulate data and hopefully in the near future I will be able to determine whether or not this technique will become a reliable indicator of preserved fossil pollen in soils.

Pollen Interpretation

Since its inception in the early 1900's the application of pollen analysis to problems of paleoenvironmental reconstruction has been constantly expanding. For the first 30 years following the establishment of the principle of pollen analysis in 1916, most pollen analyses were conducted on Quaternary sediments and were used primarily to verify established geologic assumptions (Traverse, 1971). Later, the scope of pollen analysis was widened to include not only Quaternary deposits but also studies of other Cenozoic, as well as Mesozoic and Paleozoic sediments. In the 1930's paleobotanists and petrologists began experimenting with pollen analyses of ancient sediments in England (Raistrick and Simpson, 1933) and Russia (Maumova, 1939). By the 1960's (Hopping, 1967) petroleum exploration research was also relying heavily upon fossil pollen and spore studies.

Today the scope of pollen analysis is continuously expanding and has been subdivided into numerous specialized divisions, many of which are devoted to some aspect of paleovegetation reconstruction. The remainder of this paper will focus on one specific research example in order to give the reader a clearer under-

standing of how the technique of pollen analysis can provide useable information towards the reconstruction of paleoenvironmental data.

During the late 1960's and early 1970's (Bryant, 1977c; Larson, *et al.*, 1972) I began a series of pollen analyses in an effort to reconstruct the paleovegetational history of central Texas. One of the primary locations examined was the Boriack peat bog located approximately 50 miles southwest of College Station, Texas. Radiocarbon dating of the peat deposits revealed that the earliest levels were deposited approximately 16,000 years ago and that the deposits could be used for determining vegetational changes for the area of central Texas during the last 16,000 years.

Samples were collected with a Hiller peat auger and processed using many of the techniques mentioned in this paper. Statistical counts of 200 pollen grains/sample were prepared for each level and these are seen in the form of a pollen diagram (Fig. 2). Fossil pollen data from the bottommost deposits in the peat sediments revealed a record of spruce (*Picea*), poplar (*Populus*), birch (*Betula*), hazelnut (*Corylus*), ash (*Fraxinus*), maple (*Acer*), alder (*Alnus*), dogwood (*Cornus*), pecan and/or hickory (*Carya*), basswood (*Tilia*), oak (*Quercus*), and pine (*Pinus*) in deposits radiocarbon dated as older than 15,460 ± 250 B.P. (TX-840). This suggests a central Texas vegetation during that period composed of an open deciduous woodland with some conifers present.

The exact woodland vegetation composition of central Texas during this period cannot accurately be determined from the fossil pollen record due to the differences of pollen productivity and pollen dispersion among the various species represented. However, a few salient statements can be made. Alder is one of the pollen dominants during this early period, yet its pollen productivity and dispersal is likely to make it highly overrepresented in any fossil record (Janssen, 1966). The pine and oak pollen percentages in these early deposits are not high. However, these pollen types must also be regarded as potentially overrepresented in regard to the regional importance of the plant since both are abundant pollen producers and their pollen is capable of airborne travel over great distances (Faegri and Iversen, 1975). Spruce pollen is weakly represented in these early deposits, yet it is considered underrepresented relative to the other windborne pollen types (Jonassen, 1950). Other pollen types found in these early deposits which are probably also underrepresented include basswood, poplar, and maple (Davis and Goodlett, 1960). Using these factors as considerations, I suspect that the paleovegetation of this early period most probably consisted of an open deciduous woodland forest containing some conifers and an undergrowth of shrubs and grasses.

It is difficult to infer what climatic changes would have to occur today in central Texas in order to create conditions favorable for the regrowth and survival of the proposed Fullglacial vegetation found at Boriack Bog. Since I do not know the exact species of each plant represented by the fossil pollen of those deposits, it is also risky to discuss what levels of annual precipitation or what temperature extremes may have existed in central Texas during the Fullglacial.

On the other hand, these data have been worked out for west Texas, and perhaps they can provide at least an estimate for the Fullglacial changes in central Texas. Based upon geological and hydrological studies of lake sediments on the Llano Estacado of west Texas, Reeves (1963) has suggested that it would take more than a 100% increase in present precipitation levels and at least a 73% decrease in the annual water evaporation rate to maintain the levels of west Texas lakes at their Fullglacial levels. He further concluded that the average July temperature levels in west Texas would have to be decreased by a monthly average of 9 F over what they are at present in order for those lakes to maintain their Fullglacial levels. Whether or not similar changes would also be needed to return climatic conditions in central Texas to their Fullglacial levels is not known. However, I suspect that some major climatic changes would be needed to accommodate the growth of plant genera such as spruce, hazelnut, and maple, all of which were present in central Texas during the Fullglacial period.

The fossil pollen record from Boriack and other nearby bogs suggests that between 14,000 years ago and 10,000 years ago the climatic conditions in central Texas must have continued to change towards higher annual temperatures and less available precipitation. This conclusion is inferred by the gradual degeneration of the woodland forest and the loss of all significant traces of plants such as willow, birch, spruce, maple, basswood, alder, tupelo, and ash.

During the last 10,000 years there was an apparent continual progression towards warmer and drier conditions. The gradual, but continual, loss of most tree pollen and the corresponding continual rise in grass and herb pollen during this last 10,000 year period suggests that the earlier mixed deciduous woodlands of central Texas were probably first reduced to open parklands after 14,000 years and finally reduced to the present savanna vegetation of today during the last 10,000 years.

Perhaps other researchers viewing the pollen diagram from Boriack bog might tend to interpret the paleovegetation and inferred climatic conditions somewhat differently than I have. A test to determine whether my conclusions are accurate or not would be to check them against other types of paleoenvironmental evidence derived from faunal evidence, sea level data, paleoclimatic data and/or other types of related studies. The more data that can be amassed for any given locale increases the probability that one has a more nearly accurate image of what actually has occurred.

Future Pollen Analysis

Even though pollen analysis was first developed in the early 1900's its full potential as a research tool is only now being realized. For example, in the mid-1960's Davis (1965) developed the absolute pollen frequency method for deriving more nearly accurate measurements of paleovegetation studies. Through the use of this method, palynologists are able to distinguish between vegetational changes and vegetational transitions even though the basic pollen percentages may look

alike. This is especially true of pollen records from areas near a forest-tundra boundary and areas within a forest itself. Both would show similar pollen spectra--yet the absolute pollen frequency of each area would be considerably different. The key to the absolute pollen frequency method is to accurately reconstruct the actual numbers of pollen grains that fell to earth (per unit area) at each period in the past. Recent advances in dating methods, such as radiocarbon dating, have now permitted the accurate use of this new method.

The computer has also been applied to the field of palynology, and some of the recent applications have been very useful. One of the most widely used techniques has been the multivariate method (Cole and Bryson, 1968) known as eigenvector analysis. Through the use of this method, palynological data from surface samples can be compared with fossil pollen data in such a way that quantitative reconstructions of macroclimates can be derived from fossil pollen spectra. In addition, eigenvector analysis can also be used to filter out those types of variations in pollen data which are non-climatic in origin (i.e., topography, drainage, exposure, edaphic factors).

Pollen analyses are not the "panacea" for all vegetational reconstructions. However, they do provide one useful tool in this area of study. As more data become available in the area of paleovegetation and paleoclimatic reconstruction the combined information will be useful in projects such as the proposed paleomapping of climates and weather for various regions of the world during the past. As more pollen analyses are conducted and new analytical techniques are developed, additional data will become available for interpretation and correlation.

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SOME FACTORS ASSOCIATED WITH THE DISTRIBUTION OF HELMINTHS AMONG INDIVIDUAL MOSQUITOFISH, *GAMBUSIA AFFINIS*

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ABSTRACT

The ecology of the internal helminths of *Gambusia affinis* was examined using several ecologically different habitats near San Marcos, Texas. Twenty-six species of helminths were recovered from the 1374 fish examined and 3 of these species are new North American host records. Many aspects of the helminth fauna were found to vary significantly with habitat, season and host size.

INTRODUCTION

Gambusia affinis was selected as the host species for this ecological study because: (1) it has a wide geographical and ecological distribution; (2) it occurs abundantly in many habitats; (3) it is easily collected in quantity by one person; (4) its small size allows for a short examination time; and (5) it was expected that the mosquitofish would be an important intermediate host for many piscivorous animals in its habitat because of its trophic level, omnivorous diet and surface-oriented behavior. The major objectives of this study were: (1) to identify the helminths of *G. affinis* in the study area to the extent the literature allows, (2) to study the influence of habitat differences upon this helminth fauna by comparing simultaneous collections from ecologically different habitats, and (3) to determine the extent to which the season of collection and the length and sex of the host can interact with the influence of habitat.

Most of the literature on the helminths of *Gambusia affinis* is limited to helminth surveys involving several fish hosts with little emphasis on the mosquitofish (Arnold, *et al.,* 1968; Bangham, 1941; Bangham and Vernard, 1942; Haderlie, 1953). Other works on mosquitofish internal helminths were limited to searches for single helminth species, and did not reveal pertinent information regarding helminths incidental to the searches (Bullock, 1966; Burton, 1956; Meade and

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Bedinger, 1967; Nickol and Heard, 1970). We know of no previous ecological studies that have been published on the helminths of *G. affinis,* although several works on the ecology of other fish-parasite systems have recently appeared. However, none of these studies we have seen revealed a host with such diversity of internal helminths as the *G. affinis* fauna investigated in this paper.

MATERIALS AND METHODS

Description of the Study Area

Eight collection stations were established within a study area having a radius of less than 15 km and centered near San Marcos, in southcentral Texas. The stations were selected such that fish could be examined from as many ecologically divergent habitat types as was reasonably possible. A summary of the habitat characteristics is given in Table 1 and detailed descriptions of the habitats are available in Davis and Huffman (1975).

TABLE 1

Summary of the Distinguishing Characteristics of Habitats Sampled: March, 1974 through February, 1975

Habitat	Location	Months Sampled	Fish/Sample	Characteristics
A	Pond E at SWTSU Aquatic Station.	Mar. – Feb.	25	Shallow, constant level pond.
B	Slough of Spring Lake at head of San Marcos River	Mar. – Feb.	25	Shallow; vegetation choked; stagnant.
C	Blanco River at railroad trestle below 5-mi Dam.	Mar. – Feb.	25	Shallow, high gradient hill country river; prone to drastic physicochemical fluctuations.
D	San Marcos River at Loop 82.	Mar. – Feb.	25	First order magnitude spring river; nearly constant physicochemically.
E	Little Blanco River at Route 32.	Apr., Jul., Oct., Jan.	12	Shallow hill country creek fed by small springs.
F	Canyon Reservoir at Potter's Creek Park Area.	Apr., Jul., Oct., Jan.	12	Large, deep storage reservoir on Guadalupe River.
G	Gravel pit at Junction I-35 and Loop 82.	Jun., Sep., Dec.	12	Deep to shallow gravel pit; highly eutrophic.
H	York Creek at I-35.	Jun., Sep., Dec.	12	Shallow, intermittent, lowland creek.

Collection and Examination of Hosts

The study period extended from March, 1974 through February, 1975. Twenty-five *G. affinis* were examined monthly from each of 4 "monthly collection habitats." Every effort was made to maintain uniform sampling at these 4 habitats so as to avoid introducing artificial sources of interaction between season, habitat and host length. The quantitative analyses in the study utilized only the data from these 4 monthly collection habitats.

Twelve fish were examined quarterly from each of 4 "quarterly collection habitats." These localities were visited strictly for the purpose of expanding the parasite list by sampling mosquitofish from additional habitat types.

Statistical Methods

Seasonal aspects of parasitism were analyzed using the circular distribution methods outlined in Zar (1974). Parasitologists frequently use the relatively inefficient mode (peak) of a seasonal distribution to indicate the date around which most of the parasites or infected hosts tend to cluster. Circular distribution methods allow calculating the mean date of occurrence for parasites or infected hosts. The seasonal mean is more likely to be consistent on a year-to-year basis than is the mode. This mean is symbolized by a and is actually an angle, but can be converted to a date by considering 0° as January 1 and 360° as December 31. Another very useful statistic generated by this method is r, which is a measure of the seasonal concentration of parasitism. The value of r varies from 0.0, when the data are distributed uniformly around the year, to 1.0, when all the parasites or infected fish are encountered on the same date. These statistics may be illustrated graphically by using a circle to represent the whole year and having an arrow on a radius pointing to the mean date. The length of the arrow is set to r, from 0.0 at the center to 1.0 at the circumference.

A constant number of hosts were examined each month as required by the assumptions of this method. Rayleigh's test (Zar, 1974) was employed to determine if the value of r was significant for a given sample size. Other statistical methods utilized were also after Zar (1974).

RESULTS

A total of 3,993 internal helminths representing 26 species were found in the 1,374 *G. affinis* examined. Twenty species occurred as larval forms, 3 others were present as immature adults and the remaining 3 were encountered as sexually mature adults. Helminths were found parasitizing almost every organ and tissue of the hosts. Sixty-four percent of the fish were infected with at least one helminth.

The Effects of Host Habitat

The relative abundances of each of the 22 helminth species encountered in fish from the 4 monthly collection habitats are presented in Fig. 1, with stars indicating new North American host records. Other helminth species encountered

Figure 1. Relative abundance of the helminth species commonly infecting *Gambusia affinis* from the 4 monthly collection habitats. Data are pooled over 1 yr. Eight parasite frequency classes were established with each class having twice as many parasites as the next smallest. The limits separating any 2 consecutive classes were derived by finding the number whose logarithm is halfway between the logarithms of the 2 classes. The stars refer to new host records.

were 2 unidentified species of larval nematodes from the intestine (1 specimen of each), an unidentified trematode metacercaria from the musculature, and an undescribed species of *Diplostomulum* which was common in fish from the Little Blanco River.

As can be seen from Fig. 1, the habitat from which the host was taken played a strong role in determining the composition of the helminth fauna. A "22 species by 4 habitats" contingency table analysis indicated that ecological differences between the 4 monthly habitats had a highly significant effect upon the abundance of specific helminth species [$p(X^2) < 0.001$] as well as upon the number of fish infected by each [$p(X^2) < 0.001$]. Thus, ecological differences between these geographically proximate habitats appear to have radically affected the qualitative and quantitative composition of the helminth fauna, while not perceptibly affecting the abundance of *G. affinis*. Many of these differences in parasite fauna of the habitats can be attributed to concomitant differences in the local distribution of various free living species known to serve as intermediate hosts for these parasites.

The Effects of Season

Seasonal changes influenced many aspects of the distribution of the 13 species which infected 1% or more of the 1200 fish examined from the 4 monthly collection habitats.

The seasonal variation in abundance of each of these species was found to be significantly different from a random seasonal distribution [$p(r) < 0.05$]. The mean dates of occurrence for each of these 13 species are summarized in Fig. 2,

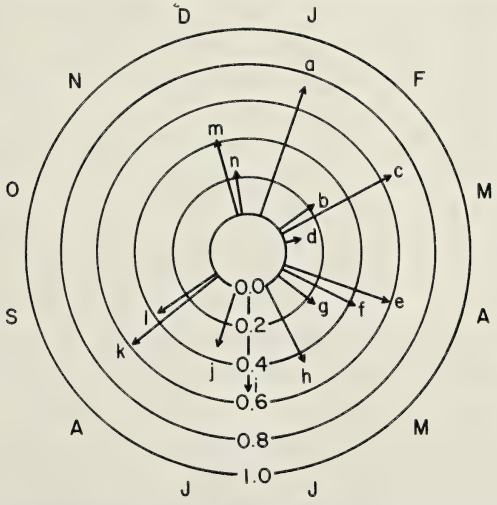

Figure 2. Seasonal concentration (r) and mean date of occurrence for each of the 13 species infecting more than 1% of the *G. affinis* from the 4 monthly collection habitats: (a) *Proteocephalus* sp.; (b) *Ornithodiplostomum ptychocheilus*; (c) *Contracaecum* sp., 2nd stage larvae; (d) *Spiroxys* sp.; (e) *Plagioporus sinitsini*; (f) *Diplostomulum scheuringi*; (g) *Crassiphiala bulboglossa*; (h) *Rhipidocotyle papillosum*; (i) *Camallanus oxycephalus*; (j) *Neoechinorhynchus cylindratus*; (k) *Phyllodistomum* sp.; (l) *Spinitectus carolini*; (m) *Contracaecum* sp., 4th stage larvae; (n) *Posthodiplostomum minimum*.

in which the arrows point to the mean dates of parasite occurrence and the length of the arrow is equal to r (outer circle: $r = 1.0$). Note how the mean dates at which the different species make their demands upon the host population appear to be temporally dispersed around the year.

Figure 3 illustrates the mean date and r value for the seasonal change in number of fish infected by each of the 13 most common species, all of which were significant [$p(r) < 0.05$], except for *Neoechinorhynchus cylindratus, Spiroxys* sp., *Posthodiplostomum minimum,* and *Ornithodiplostomum ptychocheilus.* However, when we looked at the seasonal distribution of the number of fish from the 4 habitats infected with any helminth, we found no significant seasonal changes [$p(r) > 0.5$] and a virtually constant monthly rate of infection of 67%.

The number of species found parasitizing *G. affinis* each month varied from 12 to 20, but the mean date was not significant [$p(r) > 0.5$].

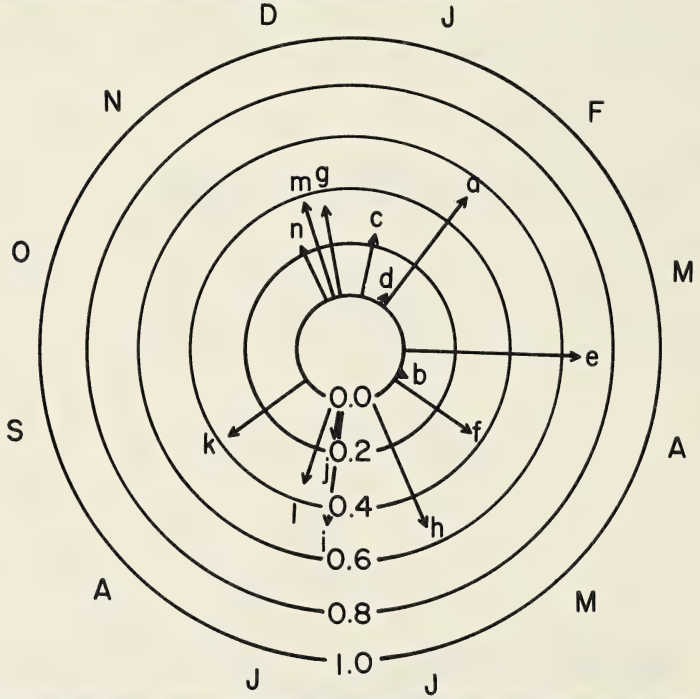

Figure 3. Seasonal concentration (r) and mean date of occurrence of fish infected with the 13 most common species infecting *G. affinis.* Species codes same as Fig. 2.

The Effects of Host Length

The standard length of the host was also strongly associated with many aspects of the *G. affinis* helminth fauna. The overall intensity increased only slightly in female fish between 15 and 30 mm, then increased more rapidly between the 30 and 45 mm length classes, beyond which intensity declined. All the individual helminth species exhibited a similar trend except for *P. minimum* and 4th stage larvae of *Contracaecum* sp., which peaked in the middle length classes.

There was also an increase in the number of helminth species with increasing standard length of host. A correction for sample size differences at the different length classes was required for this analysis since the number of species collected is generally expected to increase linearly with a geometric increase in sample size. We attempted to correct for different sample sizes at the different length classes by dividing the number of species encountered in each length class by the logarithm of the number of fish examined in that length class. The corrected mean number of species per fish increased rather linearly from 2 species for the smaller fish to about 9 species around the 30 mm length class whereupon the trend leveled off.

The % fish infected increased more or less linearly from 51% for the 146 fish examined between 14 and 19 mm standard length to 100% for the 38 fish over 39 mm. This trend was investigated by linear regression techniques by considering only those length classes in which 10 or more fish were examined and then transforming the proportion of those fish infected to arcsine of square roots. The regression for pooled sexes was significant [$p(b) < 0.001$] with a coefficient of determination of 0.75. While the slopes for the 2 sexes considered separately were not significantly different [$p(b_1 - b_2) > 0.1$], the elevations were significantly different [$p(a_1 - a_2) < 0.01$] indicating that males of a given length can be expected to have an 8% higher incidence of infection than females of the same length. This consistent difference is attributed to the greater age of the males at a given length and their consequent greater exposure to infection than females of the same length.

Additional Observations

Digenea. Diplostomulum scheuringi Hughes, 1929, was most abundant in lentic habitats and occurred as a large (1.5 mm) unencysted metacercaria in the coelom of *G. affinis,* in contrast to its usual location in the eyes of other fishes. Haderlie (1953) published the only other record of these coelomic larvae.

The forebody of this metacercaria very closely resembles the forebody of adult worms in the family Proterodiplostomatidae, all of which have been reported from crocodilians and turtles. In an attempt to find the definitive host, we collected snakes and turtles from the Aquatic Station Pond in the Spring of 1974, including 2 snapping turtles (*Chelydra serpentina*), all of which were negative for strigeoids. Holliman and Whitlock (1975) reported that snappers are definitive hosts for *D. trituri* in Mountain Lake, Virginia, and suggested that *D. scheuringi* should be synonymized with the latter. However, we believe synonymy of these species would be a premature move without much more data. We have attempted to infect snappers from our area by feeding them infected *G. affinis* and have not produced a single infection in the turtles.

D. scheuringi exhibited an April peak in abundance in this study but in subsequent years the population density of this particular worm has undergone considerable year-to-year variation, interfering with attempts to define the normal mean date. Recruitment seems to be occurring at irregular dates without detectable periodicity.

Another species of *Diplostomulum* was collected only from the Little Blanco River where it occurred abundantly in the summer and fall. This metacercaria was enclosed in a hyaline cyst of parasite origin which was in turn enclosed in a pigmented cyst in the skin of the host. This cyst differs from all known species with diplostomulum-type larvae except for the cyst of *Bulbophorus confusus,* the larvae of which differ from our specimens. Neither G. L. Hoffman (U.S.F.W.S, Stuttgart, Arkansas) nor G. L. Hendrickson (Iowa State University) could assign these *Diplostomulum* larvae to any known species.

The specimens of *Posthodiplostomum minimum* (MacCallum, 1921) collected in this study were smaller than those noted in other reports and were located in the musculature instead of the viscera. This organism was apparently not adversely affected by the characteristics of any of the habitats sampled in the study.

Our specimens of *Ornithodiplostomum ptychocheilus* (Faust, 1917) differ from Hoffman's (1960) characterization of the larvae in having a proportionately longer hindbody and a highly attenuated forebody. These worms were found encysted in the eyes of some fish and the ovaries of others. Several larger strigeoid specimens were observed free in the coelom of fish and were identified by Hendrickson as larval preencystment forms of *O. ptychocheilus.*

We were unable to identify our specimens of *Tetracotyle* sp. to species. These worms were encountered in the mesenteries of *G. affinis* from several habitats, primarily during the months of December and January. The cyst of this form is unusual in having a clear, thick gelatinous outer cyst measuring approximately 800 x 600 μm and a small, thin, inner cyst which is completely filled by the metacercaria.

Crassiphiala bulboglossa Van Haitsma, 1925, did not occur in fish from the Aquatic Station Pond but was common in black cysts in the skin of fish from the other 3 monthly collection habitats. Belted Kingfishers, the normal definitive host, were seen feeding from the pond on numerous occasions, so the absence of *C. bulboglossa* from this habitat suggests that *Helisoma anceps,* the normal molluscan host, was also absent. However, *D. scheuringi,* which Etges (1961) has reported from *Helisoma anceps* in Virginia, was abundant in the pond. Apparently, *D. scheuringi* of Texas *G. affinis* is utilizing snails other than *Helisoma anceps.*

Phyllodistomum sp. occurred in the urinary bladder of *G. affinis,* but we were unable to assign the worms to any known species due to the chaotic state of taxonomy in the genus and the substantial variability of classical taxonomic characters in our specimens. Hosts infected with *Phyllodistomum* sp. were collected mostly from the 2 springfed streams in the study, the San Marcos and Little Blanco Rivers. These 2 habitats support large populations of sphaeroid clams which serve as intermediate hosts for other species of *Phyllodistomum.*

Plagioporus sinitsini Mueller, 1934, occurred in the gall bladder of only those fish from the San Marcos River, the only habitat in which the first intermediate host, *Goniobasis* sp., was encountered. This species had a highly overdispersed distribution among hosts which resulted in an irregular seasonal distribution in

our samples. The worms were observed in varying degrees of maturity but specimens from any one fish were always of nearly equal maturity, suggesting that each infection was acquired by the ingestion of one infected snail.

Muscle cysts of *Macroderoides spiniferus* Pearse, 1924,occurred only in the *G. affinis* from the Blanco and San Marcos Rivers. The absence of this worm from the 4 smaller habitats is apparently due to the preference of the definitive host, *Lepisosteus* spp., for larger bodies of water. We did not encounter this helminth in Canyon Reservoir collections since gars occur only occasionally there and very rarely in the Potter's Creek Area of the lake where we collected our *G. affinis.*

Nematoda. Camallanus oxycephalus Ward and Magath, 1917, probably utilized *G. affinis* primarily as a paratenic host, as Spall (1968) suggested for other small fish hosts of this worm. However, at least 7 gravid female worms were encountered in the study (June–November), indicating that mosquitofish can also serve as definitive host for this nematode. The space occupied by this worm in the comparatively small rectum of *G. affinis* probably accounts for the low numbers/infected fish (1.5) despite the relatively high infection rate (11%).

Fourth stage larvae of *Contracaecum* sp. were found parasitizing the livers of many fish while apparently first (or second?) stage larvae were found in tangled masses in the mesenteries near the liver of some fish. The habitat distributions of the 2 larval stages were similar (Fig. 1), but the peak abundance of the 4th stage larvae occurred 7 to 12 months following the peak for the earlier stage (Fig. 2). An even longer time lag is seen for peak incidence of infection for the 2 forms (Fig. 3). We are not confident that the specific identity of either of these larvae can be determined from the existing literature, but the absence of a growth series suggests that the earlier stage does not develop into the later stage in one host fish.

Spiroxys sp. was the most common helminth encountered in the study and was found encysted in the mesenteries and muscles. This worm was readily identifiable to genus but we feel that specific identification of the larvae would require feeding them out to definitive hosts, which was beyond the scope of this study. This species exhibited the highest incidence of infection (37%), and yet there was an average of only 1.93 worms/infected fish, despite the absence of any signs of pathology. These observations suggest that there is a high probability that mosquitofish will encounter the infective stages of *Spiroxys* sp., but that acquired immunity or some other factor limits the success of subsequent infections for as long as the first infection remains active.

Spinitectus carolini Holl, 1928, was encountered most commonly in fish from the 2 high gradient streams, the Blanco and Little Blanco Rivers. Mayflies, which probably serve as primary hosts for this nematode, were abundant in both of these habitats.

Camallanus oxycephalus, Contracaecum sp., and *Spiroxys* sp. were most common in fish from quiet waters, probably because such habitats are more conducive to dense copepod populations than are flowing waters.

Cestoda. Small plerocercoids from the intestinal lumen of hosts from the Aquatic Station pond were assigned to *Proteocephalus* sp. instead of *Corallobothrium* on the basis of there being several largemouth bass in the pond but no catfish. *Ophiovalipora* sp. occurred as a cysticercoid in the gall bladder and was identified to genus by W. H. Coil (University of Kansas). The cestodes occurred most abundantly in fish from quiet waters probably because of the dense copepod populations which frequently develop in lentic habitats.

DISCUSSION

It appears that the quantitative and qualitative composition of the helminth fauna of *Gambusia affinis* contains substantial information regarding the local ecosystems from which the fish are collected. This is supported by the observed sensitivity of many aspects of this diverse helminth fauna to the nebulous ecological factors which characterize differing habitats. Unfortunately, many of the helminths reported in this paper cannot be reliably identified beyond the generic level. Of those that can be identified, several have incompletely known life cycles. Otherwise, one could sample the helminth fauna of *G. affinis* in an area and gain considerable information regarding the presence or absence of other hosts in the environment and, if sampling were continued, information regarding changes in the population density of those hosts could also be gained.

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EFFECT OF CYCLOHEXIMIDE AND REDUCED TEMPERATURE ON DNA REPLICATION IN CHINESE HAMSTER OVARY CELLS

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ABSTRACT

Decreased incubation temperature and inhibition of protein synthesis by cycloheximide both resulted in a reduction in incorporation of ^{3}H-thymidine into DNA in Chinese hamster ovary cells. The effect of the decreased incubation temperature appeared to be due to a reduced rate of DNA chain elongation. No effect of cycloheximide on DNA chain elongation was detected.

INTRODUCTION

Experiments with cycloheximide and other inhibitors of protein synthesis have shown that semiconservative replication of mammalian DNA is dependent upon concomitant formation of protein (Brown, *et al.*, 1970; Gautschi and Kern, 1973; Hori and Lark, 1973). Despite numerous investigations it is not clear whether continuous protein formation is required to initiate synthesis of the many replication units (replicons) comprising the mammalian genome or to maintain the rate of DNA chain elongation (Edenberg and Huberman, 1975). Hori and Lark (1973) obtained autoradiographic evidence supporting the first of these 2 alternatives. But in a similar autoradiographic study, Hand and Tamm (1973) found that blockage of protein synthesis reduced the rate of DNA chain elongation to the same extent as over-all replication. Studies using sedimentation methods to investigate the role of protein synthesis in the maintenance of replication have also yielded conflicting evidence (Hyodo, *et al.*, 1971; Gautschi and Kern, 1973; Gautschi, 1974).

Equilibrium density gradient analyses were performed in this study to compare the relative rates of DNA chain elongation in cultured mammalian cells incubated at reduced temperature or in the presence of cycloheximide. Our results indicate, as reported elsewhere (Taylor, 1968; Huberman and Horwitz, 1973), that reduced temperature decreased the rate of DNA elongation. Newly formed DNA from control and cycloheximide treated cells did not, however, appear to

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differ in length. This suggests, in contrast to the findings of Gautschi and Kern (1973) and Hand and Tamm (1973), that protein synthesis may not be required for elongation of mammalian DNA.

MATERIALS AND METHODS

Cell Line and Culture Conditions

Chinese hamster ovary (CHO) cells, clone K_1, were obtained from the American Type Culture Collection. The cells, grown in Ham's F-12 medium (Ham, 1965) supplemented with 10% fetal calf serum (Gibco), were used as asynchronous, exponentially growing monolayers in all experiments. Stock cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 in a 37 C incubator. Cultures were transferred to water baths maintained at constant temperatures (37 C or 28 C) for the experiments.

Chemicals

Cycloheximide (Acti-Dione) was a gift of Upjohn and was used at a concentration of 50 μg/ml. ^{3}H-Thymidine (^{3}H-TdR; 6.7 Ci/mmole) was purchased from New England Nuclear and ^{3}H-leucine (15 Ci/mmole) was from Schwarz-Mann. 5'-Fluorodeoxyuridine (FrdU), 5'-bromodeoxyuridine (BrdU), and RNase A were products of Sigma. Proteinase K was from E. Merck. Optical grade cesium chloride was purchased from Cal-Biochem. Amino acids and vitamins used in preparation of culture medium were purchased from Sigma and Schwarz-Mann.

Measurement of Acid Insoluble Radioactivity

Experiments illustrated in Figs. 1 and 2 were performed with subconfluent cultures 2 days after inoculation of 100 mm x 12 mm screw cap culture tubes with 1.4 x 10^5 cells each. Incorporation of precursor was stopped by washing with cold Hank's salt solution and then adding 5 ml of cold 10% trichloroacetic acid. The cells were scraped from the wall of the vessel and sonicated. Acid insoluble material was precipitated and counted as described by Gautschi and Kern (1973).

Incorporation of BrdU into DNA

Monolayers were prepared by seeding 75 cm^2 plastic flasks with 1.2 x 10^6 cells each. Two days later, the cultures were incubated in the presence of BrdU, a heavy analog of thymidine, to increase the density of replicating DNA. The basic protocol used in these experiments involved a 2 min pulse with ^{3}H-TdR (10 μCi), followed by a wash with warm Hank's solution to remove the radioactive label, and then addition of either 10 ml of medium containing BrdU (50 μg/ml) and FrdU (0.2 μg/ml) or 10 ml of plain medium (no BrdU or FrdU). The second group of cultures were given BrdU and FrdU after an interval of 5 or

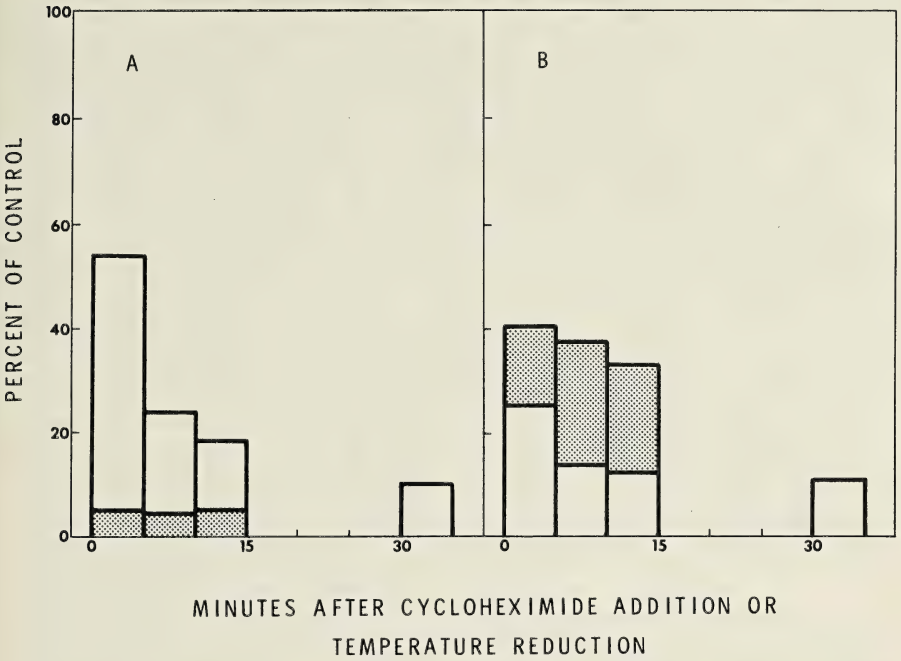

Figure 1. Inhibition of incorporation of radioactive precursors into acid insoluble products by cycloheximide and reduced temperature. Monolayers were incubated with ^{3}H-leucine (5 μCi) or ^{3}H-TdR (1 μCi) for 5 min at various times after (A) addition of cycloheximide or (B) transfer to a 28 C water bath. Results are presented relative to values obtained for control cultures and each bar is an average from 2 monolayers. ^{3}H-Leucine (▨); ^{3}H-TdR (□).

30 min. Incubation with the BrdU-FrdU mixture was for 2 hr. FrdU was used to inhibit endogenous thymidine nucleotide formation during the BrdU incorporation period. All incubations were at 37 C, except as noted.

Preparation of DNA for Centrifugation

DNA was isolated by the procedure of Gross-Bellard, *et al.*, (1973) with the following modifications: the volume of buffer A used was reduced by a factor of 4, concentrations of proteinase K and RNase A were increased by a factor of 4, and treatment with T_1 RNase was omitted. Purified DNA in buffer D (Gross-Bellard, *et al.*, 1973) was then sheared with 3 passes through a stainless steel needle.

Centrifugation in Cesium Chloride Gradients

Solutions of sheared DNA were adjusted to a density of 1.69 g/cc by addition of solid CsCl. Portions (3.8 ml) of each sample were placed in cellulose nitrate tubes (pretreated by boiling in 10 mM EDTA) and overlayed with mineral oil. Centrifugation was for 60 hr at 32,500 RPM and 20 C in a Spinco SW 50.1 rotor.

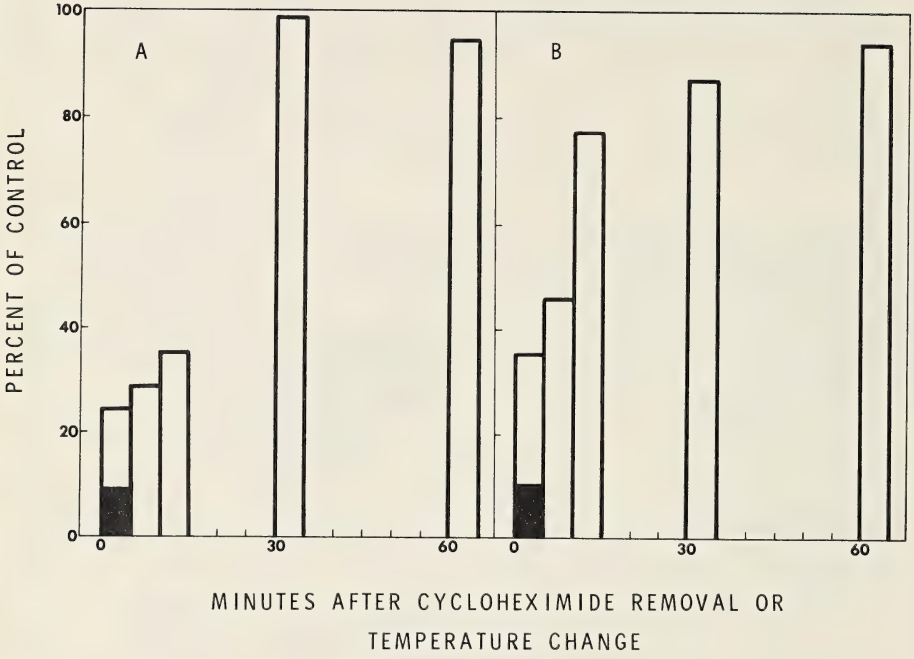

Figure 2. Reversal of the effects of cycloheximide and reduced temperature on incorporation of ^{3}H-TdR into DNA. Cultures were preincubated for 30 min with cycloheximide or at 28 C and then pulse labeled for 5 min with ^{3}H-TDR (2μCi) at various times after (A) an extensive wash to remove the inhibitor of (B) transfer from the reduced temperature to 37 C. Results are presented relative to values obtained for control cultures and each bar is an average from 2 monolayers. Solid bars represent results obtained with cultures labeled immediately after the preincubation period either (A) in the presence of cycloheximide or (B) at 28 C.

Four drop fractions were collected from the bottom of the developed gradients. Water (1.0 ml) was added to each fraction and absorbance at 260 nm was measured. Radioactivity was assayed in a mixture (1:3, v/v) of Bio-Solv (Beckman) and liquid scintillation fluid (4 g PPO and 0.05 g POPOP/1 of toluene).

Sedimentation Analysis in Neutral Sucrose Gradients

Sheared DNA samples (0.2 ml) were layered on linear gradients (5.0 ml) of 5 to 20% sucrose containing 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0), and 1 mM EDTA. An aliquot of ^{14}C labeled T_7 DNA (10 μl, 1300 CPM) was applied to a separate gradient as a standard for comparison. After centrifugation for 2 hr at 40,000 RPM and 20 C in a Spinco SW 50.1 rotor, 8 drop fractions were collected from the bottoms of the developed gradients. Water (1.0 ml) was added to each fraction and radioactivity was assayed as described above.

RESULTS AND DISCUSSION

Inhibition of Synthesis of Protein and DNA as a Function of Time after Cycloheximide Administration or Temperature Reduction

As a result of preliminary experiments conducted at various temperatures (results not shown), it was found that after a 1/2 hr preincubation at 28 C DNA synthesis was reduced to about the same extent as was observed after a 1/2 hr preincubation with cycloheximide (50 μg/ml). Experiments were then conducted to compare the kinetics of inhibition of macromolecule synthesis by cycloheximide and the reduced incubation temperature. Results of the latter experiments are presented in Fig. 1.

Addition of cycloheximide simultaneously with ^{3}H-leucine reduced incorporation of the radioactive precursor into protein to less than 6% of control (Fig 1A). Similar levels of inhibition of protein synthesis were obtained when cultures were treated with the inhibitor 5 or 10 min prior to addition of the label. DNA synthesis, however, was inhibited gradually. During the first 5 min after cycloheximide administration, incorporation of ^{3}H-TdR into DNA was only reduced to 53% of control. By 30 min after addition of the inhibitor, ^{3}H-TdR incorporation was reduced to 10% control.

During the first 5 min interval at the reduced incubation temperature, DNA synthesis decreased to 25% of control, but protein synthesis was only reduced to 41% of control (Fig. 1B). During subsequent pulse labeling intervals at 28 C, DNA synthesis was also depressed to a relatively lower level than was protein synthesis.

Recovery from Cycloheximide Inhibition and Reduced Temperature

Reversibility of the effects of cycloheximide and reduced temperature of DNA synthesis was tested with cultures preincubated for 30 min with cycloheximide or at 28 C. Following the preincubation period, monolayers were either washed repeatedly for 1 min with warm Hank's solution to remove the inhibitor or transferred from the 28 C to the 37 C water bath. The cultures were then pulse labeled for 5 min with ^{3}H-TdR at the intervals shown in Fig. 2.

The effects of cycloheximide on DNA synthesis were completely reversed within 15-30 min after washing the monolayers (Fig. 2A). Reversal of the effects of temperature on DNA synthesis was more rapid, but was incomplete (Figure 2B). Similar results were obtained in a replicate experiment.

Sucrose Gradient Analysis of Sheared DNA

The protocol chosen for analysis of the relative rates of DNA chain elongation required preparation of DNA fragments of approximately replicon size. Thus, an attempt was made to determine optimal conditions for shearing DNA. A monolayer, grown in a 75 cm^2 plastic flask, was incubated overnight with 1 μCi of ^{3}H-TdR. ^{3}H-DNA purified from the culture was divided into 3 portions.

Each portion was forced through a 21, 23, or 25 gauge needle and then layered on a sucrose gradient for sedimentation analysis. Distances that the sheared samples sedimented from the meniscus are presented in Table 1 relative to T_7 DNA (during the 2 hr centrifugation, the phage DNA sedimented slightly less than half the length of the sucrose gradient). Sedimentation coefficients of the sheared samples were estimated by multiplying their relative sedimentation distances by the S value of the standard. Molecular weights of the sheared samples were then calculated from the estimated S values using the formula of Studier (1965). As shown, there was an inverse relationship between the size of the needle used for shearing and the estimated sizes of the fragments. In a replicate experiment (results not shown), a sample sheared by passage through the 23 gauge needle sedimented with a peak coincident with that of the standard. Thus, fragment length appeared to be reproducible.

TABLE 1

Estimated Sizes of Fragments of Sheared DNA[1]

Sample		Distance sedimented relative to standard	Sedimentation coefficient (S)	Molecular weight (10^{-7})
Sheared DNA	21[2]	1.45	46.4	7.3
	23[2]	0.98	31.3	2.5
	25[2]	0.84	26.9	1.4
Standard (T_7 DNA)		1.00	32.0[3]	2.6[3]

[1] Analyses were made by sedimentation in neutral sucrose gradients.
[2] Gauge of needle used to shear DNA.
[3] Values reported by Studier (1965).

In mammalian cells, a replicon of average size is formed in approximately 20 min and at a rate of 1500–4500 deoxynucleotides/min/strand (Huberman and Riggs, 1968; Lynch, *et al.*, 1972). Using the midpoint of this range, an average sized replicon can be estimated to consist of 60,000 deoxynucleotide pairs and to have a molecular weight of approximately 3.9×10^7 daltons. DNA sheared with the 23 gauge needle appeared to be closest to this size. Thus, in the subsequent experiments, the 23 gauge needle was used for shearing.

Equilibrium Density Gradient Analysis

When a mixture of BrdU and FrdU was administered to a control culture immediately after a 2 min pulse with ^{3}H-TdR, the radioactive fragments were denser than the bulk of the cellular DNA (Fig. 3A). This indicates that BrdU was incorporated into nascent DNA in close association with ^{3}H-TdR. When BrdU and FrdU were added to a second control culture 5 min after removal of the radioactive label, less dramatic resolution of ^{3}H-DNA from bulk DNA was observed (Fig. 3B). The 5 min interval between pulse labeling and administration

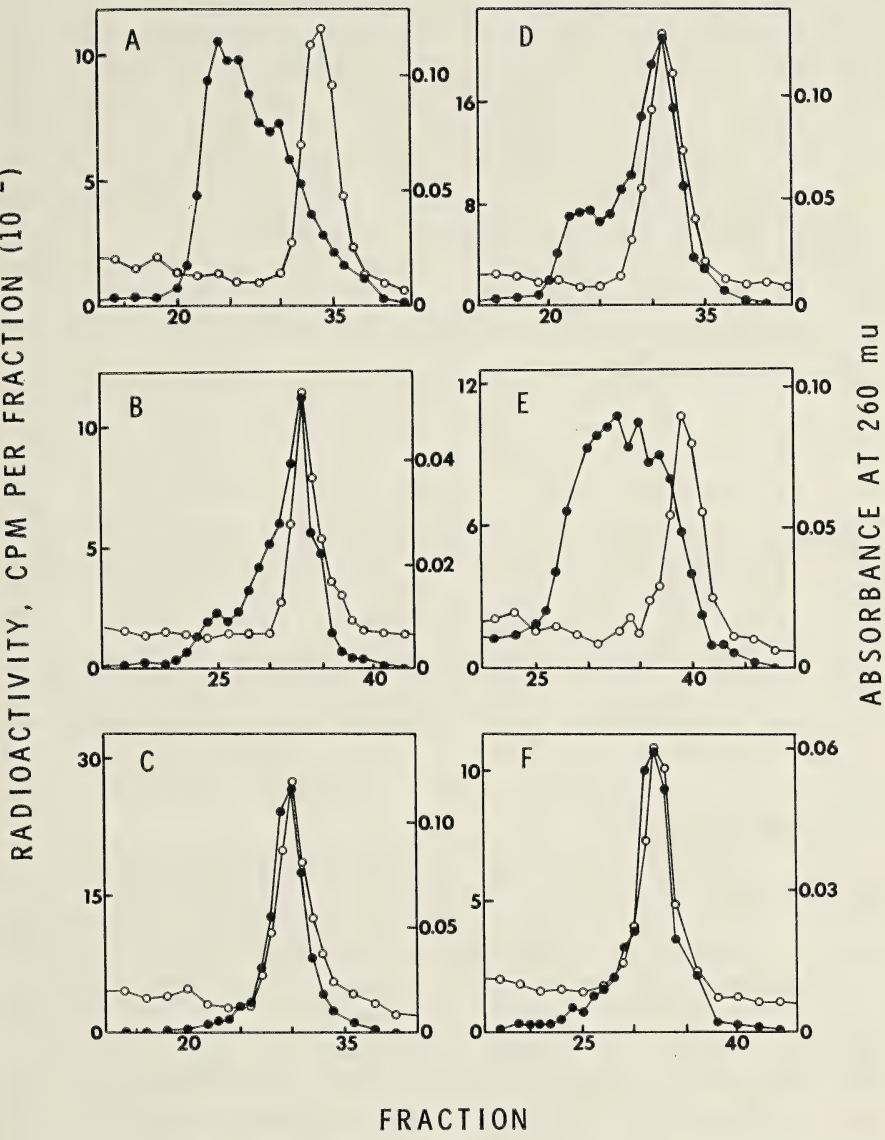

Figure 3. CsCl density gradient analysis of DNA from cultures incubated with ^{3}H-TdR and BrdU. Control cultures received BrdU and FrdU either (A) immediately, or at (B) 5 min or (C) 30 min after a 2 min incubation with ^{3}H-TdR. Another culture (D) was pulse labeled for 2 min and then incubated for 30 min at 28 C before addition of BrdU–FrdU. Cycloheximide treated cultures received BrdU and FrdU (E) immediately or (F) 30 min after the 2 min pulse period. Density increases from right to left. Fractions from the bottom and the top of the gradients (fractions 1–15 and 40–65) did not contain radioactivity and were not included in the figure. Radioactivity (•); absorbance (o).

of the BrdU-FrdU mixture would have resulted in an unlabeled, normal density deoxynucleotide chain between radioactive and BrdU-containing segments of the nascent strands of DNA. A third control received BrdU and FrdU 30 min after removal of ^{3}H-TdR. Based upon the estimated rate of polymerization (1500-4500 deoxynucleotides/min/strand; Lynch, *et al.*, 1972), it was predicted that the nonradioactive, normal density DNA chains formed during the 30 min interval between the 2 min pulse and administration of the heavy analog should be longer than the products of shearing. Thus, passage through the 23 gauge needle was expected to disrupt the association between radioactive and BrdU-containing segments of nascent DNA. Indeed, the results obtained show that BrdU was not associated with the ^{3}H-DNA fragments (Fig. 3C) and are consistent with the predictions.

Following the preliminary steps (2 min pulse, wash, and medium change), a fourth culture was transferred to a 28 C water bath. After 30 min at the reduced temperature, BrdU and FrdU were added and the culture was returned to the 37 C water bath. BrdU was associated with some of the ^{3}H-DNA prepared from this culture as evidenced by a secondary peak of radioactivity in fractions 22-24 (Fig. 3D). Similarities of the radioactive profiles shown in Fig. 3B and Fig 3D suggest that the reduced temperature decreased the rate of DNA chain elongation such that the nonradioactive, normal density DNA chains formed during the 30 min incubation at 28 C were of approximately the same length as those formed in 5 min at 37 C. The total amount of DNA that was formed in 30 min at 28 C can be estimated from Fig. 1B to have been about 1/8 of the amount formed during the same interval at 37 C. Thus, the decrease in the rate of DNA elongation at the reduced temperature was about the same as the over-all drop in DNA synthesis.

Cycloheximide was administered to 2 additional cultures 3 min prior to beginning the 2 min pulse with ^{3}H-TdR. The radioactive medium was then removed and, after a brief wash, replaced with medium containing cycloheximide. BrdU and FrdU were added to these cultures at intervals of 0 or 30 min after the medium change. Sheared ^{3}H-DNA from the culture given BrdU and FrdU immediately following removal of the radioactive precursor was denser than the bulk DNA (Fig. 3E). The other cycloheximide treated culture was subjected to a second wash step 30 min after the pulse period to remove the inhibitor (as was done with the cultures in Fig. 2A) before adding the mixture of BrdU and FrdU. Sheared ^{3}H-DNA from the latter culture was of the same density as the bulk DNA (Fig. 3F). In a replicate experiment (results not shown), ^{3}H-DNA fragments from another cycloheximide treated culture given BrdU and FrdU 30 min after the pulse period were also of the same density as bulk DNA. The apparent lack of association of BrdU with ^{3}H-DNA in Fig. 3F cannot be attributed to inhibition of incorporation into DNA. As shown in Fig 2A, cells resumed incorporation of precursor into DNA at the normal rate within 15-30 min after removal of cycloheximide.

The total amount of DNA formed during the interval between 5 and 35 min after administration of cycloheximide can be estimated from Fig. 1A to have been about 1/7 of control. Thus, if chain elongation is the only replication event dependent upon continued protein synthesis, the 2 min pulse with ^{3}H-TdR followed by a 30 min incubation in medium containing cycloheximide but no BrdU should have yielded results similar to those in Fig. 3B and Fig. 3D. If, on the other hand, cycloheximide reduced the rate of elongation of DNA by a factor of 2 or less, nonradioactive, normal density segments of approximately 45,000 deoxynucleotide pairs, or 2.9 x 10^{7} daltons (similar to the size of the fragments of sheared DNA), would have been formed during the 30 min interval between the pulse period and administration of BrdU-FrdU and no resolution of ^{3}H-DNA from bulk DNA would have resulted. Our results are consistent with the latter alternative.

CONCLUSION

At the decreased incubation temperature the rate of DNA chain elongation was reduced to about the same extent as the over-all reduction of DNA synthesis. But, the cycloheximide treatment either did not decrease the rate of DNA chain elongation or did so by an amount insufficient to account for its dramatic inhibition of replication. This suggests that administration of the inhibitor may have interfered with initiation of new points of replication.

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DIFFERENCE IN DIAMINE OXIDASE (DAO) ACTIVITY OF PLASMA AND SERUM OF PREGNANT WOMEN

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ABSTRACT

This study reveals the comparison of diamine oxidase (DAO) activity between serum and ethylenediaminetetraacetic acid (EDTA) plasma of pregnant women. DAO activity of EDTA plasma is shown to be 8%–18% lower than serum. When DAO activity of heparinized plasma is compared with serum of pregnant women no difference in DAO activity is observed. These data indicate the interference of EDTA and suggest that either serum or heparinized plasma of pregnant women be used for DAO determination.

INTRODUCTION

It has been reported by Weingold (1968) that diamine oxidase (DAO) is present in the maternal blood of pregnant women but is not detected in the blood of men or non-pregnant women. Moreover, a review of the literature indicates other enzymes such as oxytocinase and alkaline phosphatase heat stable at 56C have also been detected in the maternal blood of pregnant women but absent in men and non-pregnant females. These enzymes are suspected to originate in the placenta. Hansson, *et al.*, (1969) have shown that DAO is in the blood plasma and lymph of men and non-pregnant women who have received intravenous heparin. Hampton and Pavelee (1969) have reported the DAO activity in heparinized plasma from marmosets did not differ between males, pregnant females, and non-pregnant females marmosets. From these studies, it appears that there is a factor which causes the release of DAO into the blood. Indeed, heparin may be the factor which is responsible for the release of DAO in pregnant women and male and female marmosets.

It has been proposed by Okuyama and Kobayashi (1961) that the physiological function of DAO is to degrade histamine during pregnancy. The enzyme *in vitro* is involved in the oxidation of diamines such as putrescine and cadaverine although these substances have yet not been identified in biological tissues. Putrescine and cadaverine are decarboxylation products of the amino acids ornithine and lysine respectively.

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Weingold (1968) has reported that DAO activity normally becomes measurable at the 8th week of pregnancy, and there is almost a linear rise during the first 20 weeks of pregnancy. Enzyme activity levels off until the term. Although there is a wide variation in enzyme activity in normal pregnant females, extremely low levels of DAO have been reported during threatened, habitual and missed abortion and in diabetic patients. There does not, however, appear to be a correlation between low levels and fetal outcome.

Usually, the specification of either plasma or serum samples is necessary for accurate analysis and to avoid interferences. For example, serum rather than plasma samples are required for the radioimmuno assay of prostaglandin, insulin, and follicle stimulating hormone (FSH). It has been noted in the cited literature, however, that some investigators have analyzed DAO activity in plasma while others use serum. However, no difference in activity between serum and plasma has been mentioned. The primary objective of this study was to compare DAO activity in the plasma and serum of pregnant women.

MATERIALS AND METHOD

Samples of serum and plasma with EDTA as the anticoagulant were collected from 5 pregnant women. Samples of serum and heparinized plasma were collected from 5 other pregnant women for DAO analysis. Assays were performed in duplicate on plasma and serum samples by the radiochemical method of Hampton and Parmelee (1969). In the general procedure, an excess and a measured amount of radioactive and non-radioactive substrate putrescine C^{14} is reacted with a known volume of either serum of plasma for 2 hr at 37 C on a metabolic shaker. The product formed by the reaction is extracted in scintillation fluid containing the end product of putrescine metabolism is then decanted from the frozen aqueous phase containing unreacted substrate and delivered to a counting vial. The counts per min/vial were quantitated for 5 min in a liquid scintillation counter. Activity is expressed as "Units of DAO causing the disappearance of 0.01 μg of putrescine/ml plasma or serum/2 hr incubation.

RESULTS AND CONCLUSION

The analysis of each sample was carried out, and the average of each analysis is shown in Table 1. Counting error of the liquid scintillation spectrophotometer (Beckman LS-150) was in the range of 0.5% to 1.0%. This study indicates that the DAO activity in EDTA plasma is significantly less (8%–18%) than that measured in heparinized plasma or serum ($p < 0.05$). It has been reported by West, *et al.*, (1966) that a number of enzymes like lipase, succinic dehydrogenase, adenosine triphosphatase and certain proteolytic enzymes are activated by inorganic Ca^{+2} as cofactor. It is possible that DAO requires an inorganic ion as cofactor. The inorganic ion might have been chelated by EDTA accounting for the lower DAO

TABLE 1

DAO Units of Serum, EDTA Plasma and Heparinized Plasma of Pregnant Women

Subject No.	Gestation in Weeks	DAO Units		
		Serum	EDTA Plasma	Heparinized Plasma
1	26	4282	3653	--
2	24	662	562	--
3	42	2336	2186	--
4	36	6581	5640	--
5	40	6335	5377	--
6	26	996	--	996
7	20	883	--	893
8	36	2032	--	2156
9	28	1104	--	1099
10	18	823	--	831

activity. It is also possible that lower DAO activity in EDTA plasma may be accounted for by interaction of EDTA with putrescine. No significant difference in DAO activity was detected between heparinized plasma and serum. This investigation suggests that either serum or heparinized plasma should be used for DAO determination.

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APPLICATION OF GROUP ENTHALPIES OF TRANSFER IN TRANSITION STATE ENTHALPY OF TRANSFER STUDIES[1]

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ABSTRACT

The rate of the reaction of p-acetylbenzyl chloride, p-acetylbenzyl bromide, p-methoxybenzyl chloride, p-methoxybenzyl bromide, p-cyanobenzyl chloride, and p-cyanobenzyl bromide with pyridine in methanol and dimethylformamide (DMF) were determined at 25 and 50 c. The heats of solution of the reactants in these 2 solvents were calculated using the additivity principle of Fuchs, *et al.*, (1973). From these data the transfer enthalpies of the transition states of the reactions from methanol to dimethylformamide were calculated. Greater solvation of the transition states in the dipolar aprotic solvent rather than desolvation of the reactants is the cause for the observed lower activation enthalpy in the dipolar aprotic solvent.

INTRODUCTION

The observed dramatic increases in rates of S_N2 reactions with a corresponding consistent decrease in the enthalpy of activation ($\Delta H^{\ddagger}$) on change from a polar protic to a dipolar aprotic solvent has been the subject of thorough studies within the last ten years (Parker, 1968; Abraham, 1969; Haberfield, *et al.*, 1971; Fuchs and Cole, 1973).

It appears that these changes in reaction rate and activation enthalpy can be attributed to either (a) an increase in ground-state energy by desolvation of reactants in the dipolar aprotic solvent, or (b) a decrease in transition state energy by increased solvation of the activated complex in the dipolar aprotic solvent. At the same time it is also conceivable that a combination of both effects, (a) and (b), will give the same results.

The changes in rate and activation enthalpy then are due to differences in solvation enthalpies and not to entropy differences (Parker, 1968). The result is that direct analysis of solvent effects is possible by calculating the enthalpies of transfer of the reactants and transition state (Arnett, *et al.*, 1965; Arnett and McKelvey, 1966; Haberfield, *et al.*, 1969; Fuchs and Cole, 1973) the latter being obtained from the relation

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$$\Delta\Delta H^t = \Delta\Delta Hs + \Delta\Delta H^{\ddagger}$$

where $\Delta\Delta H^{\ddagger}$ is the difference in the activation enthalpies of the reaction in the 2 solvents, $\Delta\Delta Hs$ is the enthalpy of transfer of the reactants from one solvent to the other, and $\Delta\Delta H^t$ is the enthalpy of transfer of the transition state.

The purpose of the present work is to demonstrate the application of group enthalpies of transfer in such studies. Group enthalpies of transfer are a recent development in studies of solvent - solute interactions (Fuchs, *et al.*, 1973; Fuchs and Rodewald, 1973; Krishman and Friedman, 1971). The use of group enthalpies of transfer values eliminates the calorimetric determinations that are required to obtain values of $\Delta\Delta Hs$ of the reactants.

It was reported (Fuchs, 1974) that deviations from additivity occur with group enthalpies of transfer only when there exists a possibility for intramolecular hydrogen bonding (o-nitroaniline and o-nitrophenol) and also in the case of a series of m- and p- substituted benzaldehydes where uncatalyzed hemiacetal formation appears to occur in methanol. The para substituted benzyl halides in the present study however are believed to be incapable of exhibiting these kinds of behavior.

EXPERIMENTAL RESULTS

The solvent and p-substituent effects upon the rates of the reaction

$$p\text{-}YC_6H_4\cdot CH_2\cdot X + C_5H_5N \rightarrow p\text{-}YC_6H_4\cdot CH_2\cdot NC_5H_5^+ + Cl^-$$

in methanol and dimethylformamide at 25 and 50 C can be seen in Table 1. The results of the reaction of the 6 para substituted benzyl halides with pyridine coincide with and are supported by other reports in the literature (Abraham, 1969; Haberfield, *et al.*, 1971) concerning studies of benzyl halides and amines under similar conditions. The thermodynamic activation parameters are listed in Table 3. The rate increases are reflected in a substantial lowering of the $\Delta H^{\ddagger}$ in DMF.

Values of $\Delta\Delta Hs$ were determined for the 6 p-substituted benzyl halides (Table 2) using values reported by Fuchs, *et al.*, (1973). For example, an enthalpy of transfer value of -0.97 kcal/mole was calculated for p-methoxybenzyl chloride using the following group enthalpies of transfer in kcal/g group wt: Cl, -0.1; CH_3O, -0.6; alpha-CH_2, 0.03; and C_6H_4, -0.3. Enthalpy of transfer values, $\Delta\Delta Hs$, were determined for the other p-substituted benzyl halides using the method as described above. The enthalpy of transfer of pyridine was determined experimentally in the 2 solvents at 25 C.

Values of $\Delta\Delta H^t$ (Table 2) when compared with corresponding values of $\Delta\Delta Hs$ for each reaction indicate that the substantial decreases in the enthalpy of activation on going from methanol to DMF are caused by enhanced solvation of the transition state in DMF rather than by desolvation of the reactants. This is of course in tune with general findings in studies of Menshutkin type reactions (Abraham, 1969; Haberfield, *et al.*, 1971).

TABLE 1

Rates of Reaction of Pyridine with p-Substituted Benzyl Halides

Reactants	Solvents	Temp (C)	$k_2 \times 10^5$ [a]
p-Cyanobenzyl chloride	MeOH	25	0.167 ± 0.0024
	DMF	25	0.271 ± 0.0040
	MeOH	50	3.630 ± 0.0200
	DMF	50	2.800 ± 0.0600
p-Acetylbenzyl chloride	MeOH	25	0.298 ± 0.0050
	DMF	25	0.331 ± 0.0050
	MeOH	50	3.930 ± 0.0800
	DMF	50	2.590 ± 0.0400
p-Methoxybenzyl chloride	MeOH	25	1.300 ± 0.0190
	DMF	25	1.510 ± 0.0300
	MeOH	50	10.300 ± 0.2700
	DMF	50	7.070 ± 0.0400
p-Cyanobenzyl bromide	MeOH	25	5.790 ± 0.0500
	DMF	25	63.300 ± 0.7000
	MeOH	50	46.300 ± 0.8000
	DMF	50	362.400 ± 7.1000
p-Acetylbenzyl bromide	MeOH	25	8.410 ± 0.0600
	DMF	25	80.400 ± 0.3900
	MeOH	50	51.200 ± 0.7000
	DMF	50	381.600 ± 2.4000
p-Methoxybenzyl bromide	MeOH	25	43.600 ± 0.9000
	DMF	25	188.000 ± 3.5000
	MeOH	50	190.000 ± 2.6000
	DMF	50	729.000 ± 2.6000

[a] liters/mole sec. Error limits are standard deviations (see text).

TABLE 2

Enthalpies of Transfer[a] from Methanol to Dimethylformamide at 25 C

Reactants	$\Delta\Delta Hs$[b]	$\Delta\Delta H^{\ddagger}$	$\Delta\Delta H^{t}$
p-Cyanobenzyl chloride	-0.56 ± 0.3	-5.7 ± 0.3	-6.26 ± 0.3
p-Acetylbenzyl chloride	-0.56 ± 0.2	-5.5 ± 0.4	-6.06 ± 0.4
p-Methoxybenzyl chloride	-0.16 ± 0.4	-4.0 ± 0.2	-4.16 ± 0.4
p-Cyanobenzyl bromide	-0.66 ± 0.3	-2.6 ± 0.3	-3.26 ± 0.3
p-Acetylbenzyl bromide	-0.66 ± 0.3	-1.9 ± 0.3	-2.56 ± 0.3
p-Methoxybenzyl bromide	-0.26 ± 0.4	-0.9 ± 0.2	-1.16 ± 0.4

[a] kcal/mole

[b] Values represent the sum of $\Delta\Delta$ Hs(pyridine) + $\Delta\Delta$Hs(p-substituted benzyl halides). The $\Delta\Delta$Hs of pyridine is 0.81 kcal, obtained experimentally using the method described by Fuchs, *et al.*, (1973).

TABLE 3
Activation Parameters for Pyridine – p-Substituted Benzyl Halide Reactions

Reactants	Solvent	$\Delta H^{\ddagger a}$	$\Delta S^{\ddagger}$, eu	$\Delta G^{\ddagger a}$
p-Cyanobenzyl chloride	MeOH	22.9 ± 0.1	- 8.10	25.31
	DMF	17.2 ± 0.2	-26.30	25.04
p-Acetylbenzyl chloride	MeOH	20.6 ± 0.1	-14.70	24.98
	DMF	15.1 ± 0.3	-32.40	24.75
p-Methoxybenzyl chloride	MeOH	15.2 ± 0.1	-29.90	24.10
	DMF	11.2 ± 0.1	-42.90	23.90
p-Cyanobenzyl bromide	MeOH	15.3 ± 0.1	-26.60	23.20
	DMF	12.7 ± 0.2	-30.50	21.80
p-Acetylbenzyl bromide	MeOH	13.2 ± 0.2	-32.90	23.00
	DMF	11.3 ± 0.1	-34.70	21.60
p-Methoxybenzyl bromide	MeOH	10.6 ± 0.1	-38.30	22.01
	DMF	9.7 ± 0.1	-38.50	21.10

[a]kcal/mole

Values of $\Delta\Delta$Hs in Table 2 represent the sum of $\Delta\Delta$Hs of pyridine (0.81 kcal/mole) and $\Delta\Delta$Hs of the benzyl halides from 1 solvent to the other. Clearly, the endothermic $\Delta\Delta$Hs of pyridine is cancelled by nearly equal exothermic $\Delta\Delta$Hs values of the benzyl halides. Thus the values of $\Delta\Delta$Hs in Table 2 are nearly zero.

Table 4 shows enthalpies of transfer of S_N2 and S_NAr reactions from methanol to DMF reported by Haberfield, *et al.*, (1969). We have calculated $\Delta\Delta$Hs values of the substrate reactants using appropriate group enthalpies of transfer which gave the resulting transition state enthalpies of transfer as seen in Table 5. Differences in the values seen in Table 4 and Table 5 are small. The important observation to be made is that there is good agreement between calculated $\Delta\Delta$Hs values in Table 5 and observed $\Delta\Delta$Hs values in Table 4 for all the reactions with the exception of the 2,4-dinitroiodobenzene – SCN^- reaction where a deviation of 0.9 kcal exists. It should be pointed out that Fuchs and Rodewald (1973) report a deviation of 0.4 kcal in the calculated and the observed $\Delta\Delta$Hs values of 2,4-dinitroiodobenzene and further attributed this to experimental error.

METHOD OF RATE DETERMINATION

The concentrations of the benzyl halides and pyridine were equal in each reaction, ranging from 0.01 to 0.40 M. Rates were determined by following the appearance of halide ion and also by the disappearance of base as an added check. The base concentration was determined by titration with perchloric acid in glacial acedic acid solvent. Halide ion concentration was determined by adding an aliquot of the reaction mixture. to a mixture of ice water and ligrion in a separatory funnel. After several washings, the aqueous phases were titrated for halide ion using the Volhard method. The second-order

TABLE 4[a]

Enthalpies of Transfer[b] of S_N2 and S_NAr Reactions from Methanol to Dimethylformamide at 25 C

Reaction	$\Delta\Delta Hs$	$\Delta\Delta H^{\ddagger}$	$\Delta\Delta H^{t}$
$SCN^- + CH_3I$	-4.00	-4.30	-8.30
2,4-Dinitrophenoxide + CH_3I	-0.69	-6.40	-7.10
4-Nitrophenoxide + CH_3I	3.59	-7.70	-4.10
SCN^- to 2,4-dinitroiodobenzene	-6.80	-0.45	-7.25
SCN^- + 2,4-dinitrochlorobenzene	-5.26	-3.08	-8.34
N_3^- + 4-nitroiodobenzene	-1.36	-5.60	-7.00
N_3^- + 4-nitrofluorobenzene	-0.95	-4.80	-5.80

[a]Data from Haberfield, *et al.*, (1969).
[b]kcal/mole

TABLE 5[a]

Enthalpies of Transfer[b] of S_N2 and S_NAr Reactions from Methanol to Dimethylformamide at 25 C

Reaction	$\Delta\Delta Hs$[c]	$\Delta\Delta H^{\ddagger}$	$\Delta\Delta H^{t}$
$SCN^- + CH_3I$	-3.87	-4.30	-8.20
2,4-Dinitrophenoxide + CH_3I	-0.56	-6.40	-7.00
4-Nitrophenoxide + CH_3I	3.72	-7.70	-4.00
SCN^- + 2,4-dinitroiodobenzene	-5.90	-0.45	-6.35
SCN^- + 2,4-dinitrochlorobenzene	-5.35	-3.08	-8.43
N_3^- + 4-nitroiodobenzene	-1.14	-5.60	-7.00
N_3^- + 4-nitrofluorobenzene	-0.78	-4.80	-5.60

[a]Data from Table 4 with values of $\Delta\Delta Hs$ calculated from group enthalpies of transfer (Fuchs, 1972), thus resulting in the observed values of $\Delta\Delta H^{t}$.
[b]kcal/mole
[c]Values represent the sum of $\Delta\Delta Hs$ values of ionic nucleophiles (Haberfield, *et al.*, 1969) and $\Delta\Delta Hs$ values of reactive substrates obtained from appropriate group enthalpies of transfer (Fuchs, *et al.*, 1973).

rate constants are the results of 3 or more runs. The standard deviations are listed in Table 1. Attempts to determine the rate constants using the second-order rate equation resulted in a constant upward drift for several reactions studies. Similar results were reported by Haberfield, *et al.*, (1971) and were attributed to concurrent solvolysis. We therefore treated our data by the method reported by Young and Andrews (1944) using the expression

$$dx/dt = k_1(a - x) + k_2(a - x)(b - x)$$

Values of k_2 were obtained by plotting $(dx/dt)/a - x$ against $b - x$ which gave a straight line. The slope of this line is k_2. We calculated a second-order rate constant of 3.08×10^{-5} 1/mole sec. for the reaction of p-nitrobenzyl chloride with pyridine in DMF at 50 C. This agrees quite well with that reported by Haberfield, *et al.*, (1971), 2.64×10^{-5} 1/mole sec.

ERRORS

The standard deviation of each set of rate constants at a given temperature was obtained by a least-squares treatment of the data employing a STATPAC X-7 computer system (Table 1).

The errors reported for $\Delta\Delta H^{\ddagger}$ are the result of the individual errors in the corresponding values of $\Delta H^{\ddagger}$ obtained by calculating maximum and minimum values of the slope from the equation

$$\Delta H^{\ddagger} = \frac{RTT'}{T' - T} \ln \frac{k'T}{k\,T'}$$

using standard deviations in rate constants and the accuracy of $\pm 0.01^{\circ}$ in temperature measurements. Deviations in the values of $\Delta\Delta H^{t}$ represent the estimated error, derived by a complete treatment of all primary errors.

Activation entropies, $\Delta S^{\ddagger}$, were determined from $k = (eKT/h) \exp(-Ea/RT) \exp(\Delta S^{\ddagger}/R)$, and at a given temperature, the energy of activation $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\,\Delta S^{\ddagger}$.

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MASS SPECTRAL STUDIES OF FLUORENONE AND FLUORENE

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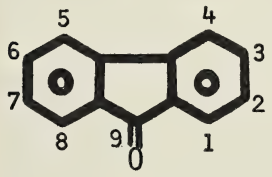
ABSTRACT

A series of deuterium-substituted and ^{13}C-labeled fluorenone (1) and fluorene (2) derivatives were synthesized and studied by mass spectrometry. The series were fluorenone (1a), *carboxyl*-^{13}C-fluorenone (1b), 2-deuteriofluorenone (1c), 4-deuteriofluorenone (1d), fluorene (2a), C9-^{13}C-fluorene (2b), 2-deuteriofluorene (2c), and 4-deuteriofluorene (2d). The fragmentation patterns for these compounds indicate that fluorenone loses CO and then acetylene; and that fluorene retains the C-9 carbon, experiences β-cleavage (analogous to an alkylbenzene), and rearranges to form tropylium derivatives.

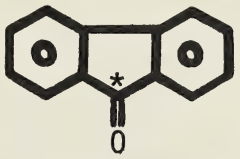
INTRODUCTION

Whereas aliphatic compounds generally experience extensive decomposition in mass spectral processes, aromatic compounds exhibit a relatively small amount of fragmentation (Budzikiewicz, *et al.*, 1967). Thus, relatively little work has been done in the field of polynuclear aromatic hydrocarbons, and this has led to uncertainty regarding the types of mechanistic decompositions that occur.

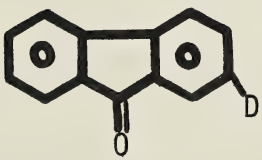
In the present account, a mass spectral study is described of fluorenone (1) and fluorene (2). In this study, various C-13 and deuterated derivatives of 1 and 2 were synthesized (see structures 1a-d and 2a-d). The fluorenone/fluorene



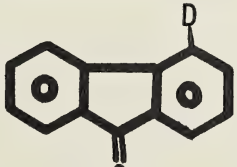
1a



1b (C-13 at indicated position)



1c



1d

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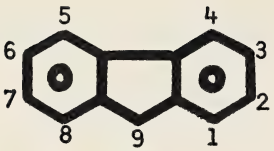
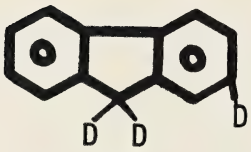
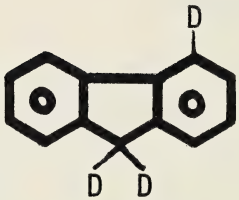


2a

2b (C-13 at indicated position)



2c



2d

series of compounds was chosen because (1) it was desired to have no alkyl or other substituents, since fragmentations at the substituent would predominate; and (2) nevertheless *internal* aliphatic bridging would be an interesting variation of structure that might clarify reorganizational processes (such as ring expansion) in polynuclear aromatic compounds (Budzikiewicz, *et al.*, 1967).

Mass spectral data for compounds 1a-d and 2a-d are given in the Experimental.

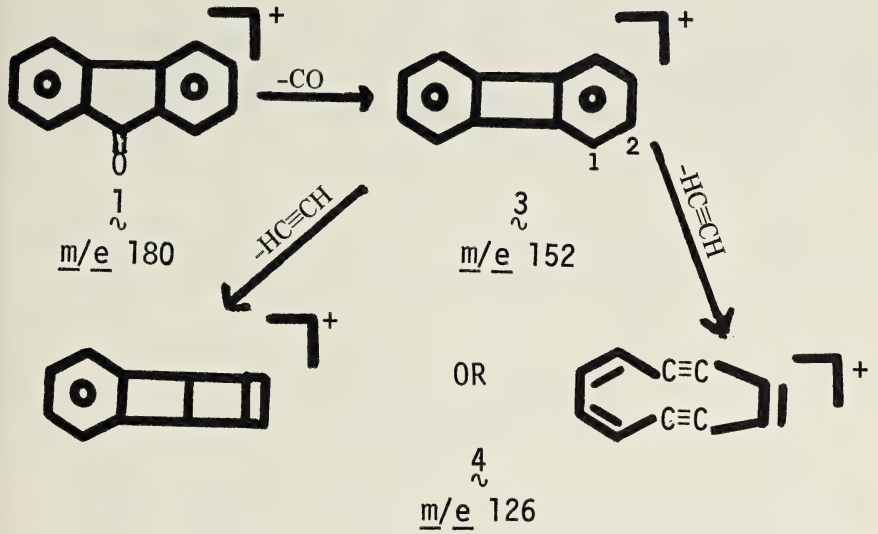
Fluorenone (1)

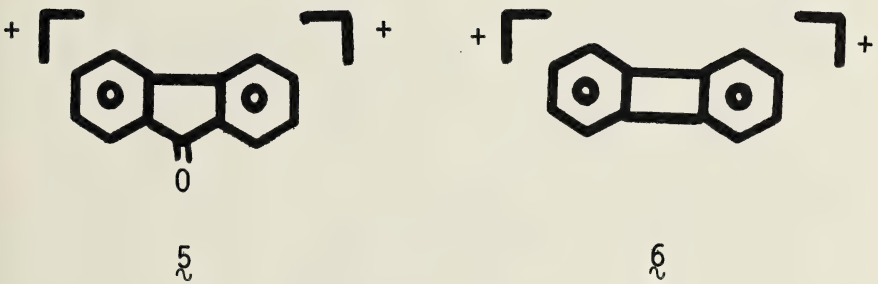
The mass spectral peak at *m/e* 152 is due to the loss of CO, shown by the loss of an extra atomic unit from C-13 labeled 1b. The resulting compound is indicated to be biphenylene (3), since anthraquinone loses two CO molecules to give biphenylene (Beynon, *et al.*, 1959). Since the spectra of 1a and 1b are virtually identical below the parent peak region, it is apparent that this loss of CO precedes all other reactions and thus is the first step to all fragmentations.

The next spectral region (*m/e* 126) corresponds to the loss of HC≡CH (acetylene) from 3. The fact that the deuterium can be lost from both compounds 1c (where the D is β) and 1d (where the D is α) shows the lost acetylene must include both C-1 (or C-4, C-5, C-8) and C-2 (or C-3, C-6, C-7) of 3. Thus, the loss of acetylene involves C-1, C-2 as shown to give 4.

The origin of the peaks at *m/e* 98–102 is more obscure. The fact that the deuterium is retained part of the time for both 1c and 1d shows that, as for 4, both C-1 and C-2 must be lost.

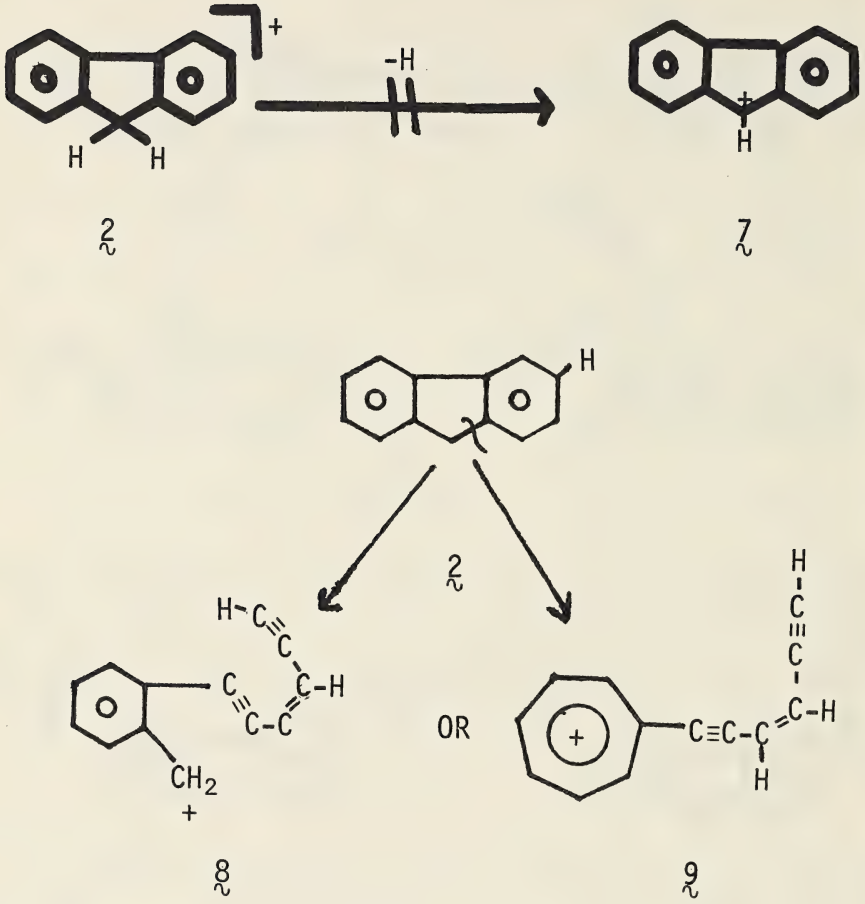
The peak at *m/e* 90 corresponds to the double ion 5, shown by peaks at *m/e* 90-1/2 for 1b-d. Likewise, the peak at 76 corresponds to the double ion 6, shown by the peaks at 76 for 1b and 76-1/2 for 1c-d.



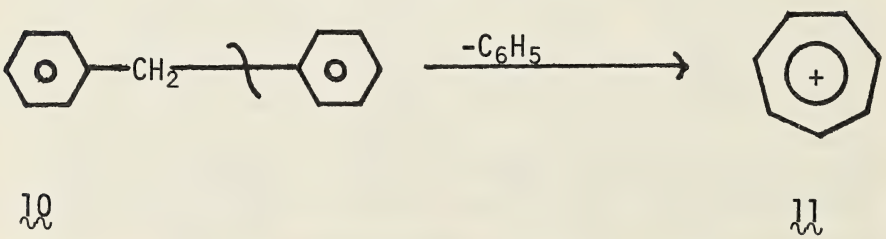


Fluorene (2)

The P-1 peak for 2a shows loss of a hydrogen, and one might anticipate the creation of the stable fluorenylcation 7 by loss of the 9-H hydrogen. However, since 2c and 2d do not show a strong P-2 peak, and since the P-1 peak is virtually the same for all compounds 2a-d, then the process shown to give 7 must *not* be the case. Instead, one of the aromatic hydrogens is lost. Study of the spectral patterns of 2c and 2d also shows that H-2 or H-4 is not lost. A mechanism rationalizing this result is readily available once it is remembered that alkylbenzenes commonly undergo β-cleavage (Grubb and Meyerson, 1963). Accordingly, it is proposed that this ion arises by β-cleavage and loss of H-3 to give 8. Since diphenylmethane (10) ejects a phenyl group to form tropylium (11) (Eland and Danby, 1965), then the analogous process for 2 would actually give 9, a tropylium ion species.

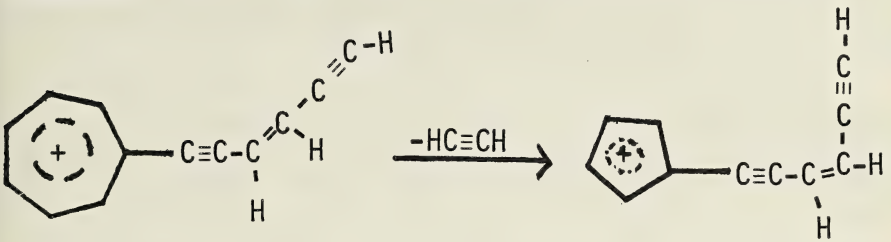


The fragment *m/e* 139 corresponds to loss of C_2H_3. Study of 2b shows the C-9 carbon is retained, and those of 2c and 2d show 1 deuterium sometimes is



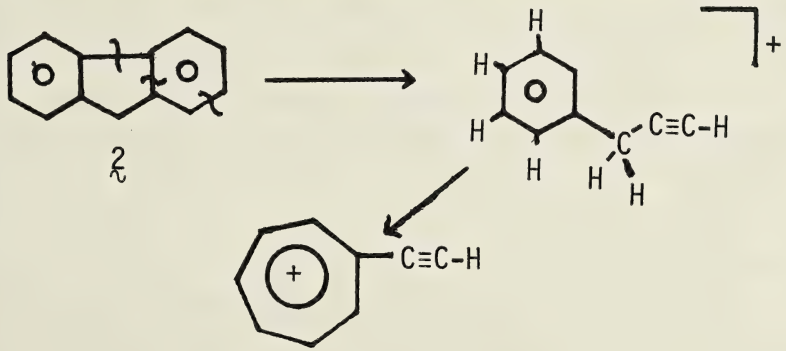
lost. Since tropylium is known to lose acetylene to form cyclopentadienyl cation (Budzikiewicz, *et al.*, 1967) the ion 9 could do likewise to form 12. Since the

tropylium ring has been fairly well rearranged (in fact, tropylium may itself be a rapidly equilibrating structure (Grubb and Meyerson, 1963)), then the loss of deuterium from 9 to give 12 would be fairly indiscriminate, consistent with what is observed.



9 12

The fragment *m/e* 115 corresponds to C_9H_7. Again, the spectrum of 2b shows that C-9 is retained, and those of 2c,d show that usually two deuteriums are retained. It is difficult to see how such a fragment could arise from 9 or 12. However, if initial cleavage occurs at the other (the direct) bridge between the two phenyl rings, then tropylium can arise in a different manner (see 13).



2 13

The double ions at 83, 82-1/2, and 82 correspond to the single ions 166, 165, and 164. The double ions for 2b-d also correspond to the P, P-1, and P-2 ions.

EXPERIMENTAL

Synthesis of ^{13}C-Labeled Compounds

Synthesis of ^{13}C-labeled compounds with isotopic enrichment of >90% utilized carbon dioxide–^{13}C (Monsanto Corp., Miamisburg, Ohio) with vacuum-line techniques previously reported (Marshall and Miiller, 1973).

Diphenyl-2-Carboxylic Acid-^{13}C

Diphenyl-2-carboxylic acid-^{13}C was synthesized by using the previously published procedure (Heidelberger and Rieke, 1951) for the ^{14}C-analog, incorporating the ^{13}C-label by means of > 90%-^{13}C-carbon dioxide. The reaction mixture was worked up in the usual manner (Marshall and Miiller, 1973), giving a white crystalline solid 2.9 g (66%, based on the carbon dioxide).

Fluorenone-9-^{13}C

Fluorenone-9-^{13}C was synthesized by using the previously published procedure (Heidelberger and Rieke, 1951) to give a greenish-yellow product, 0.71 g (98% yield).

Fluorene-9-^{13}C

Fluorene-9-^{13}C was synthesized by using the previously published procedure (Harris, *et al.*, 1955) to give 0.4 g (89% yield).

2-Deuterio-9-Fluorene

A 25 ml roundbottom flask was purged with nitrogen gas and immersed in a water bath at 20 C. 2-Bromo-9-fluorene (0.50, 2 mmol) (Aldrich Chemical Company, Milwaukee, Wisconsin) was dissolved in 14.0 ml of methanol-O-D and then solid palladium chloride (0.72 g, 4 mmol) was added (Bosin, 1973). Solid sodium borodeuteride (99%D) (0.836 g, 20.0 mmol) was then added in small portions to the stirred solution and stirring was continued for 1 hr. The reaction mixture was poured into 160 ml of water containing 10 ml of 5 *N* HCl. Extraction of the aqueous phase with 3 X 30 ml of ethyl ether, drying the combined ether extracts with anhydrous $MgSO_4$, filtration, and removal of the ether under reduced pressure, yielded 0.31 g (91%).

2-Deuterio-9-Fluorenone

2-Deuterio-9-fluorene (0.25 g, 1.45 mmol) was dissolved in solvent-grade benzene (2 ml) in a 25 ml Erlenmeyer flask which was fitted with a magnetic stirring bar. To this was added solid potassium hydroxide (0.125 g) and 18-crown-6 (200 mg) (Durst, 1977). The solution was vigorously stirred, and opened to the atmosphere for 1 hr. The benzene was decanted into a separatory funnel and washed with 6 *N* HCl (5 ml) and saturated brine (2 X 5 ml), treated with charcoal, and dried over anhydrous $MgSO_4$. Filtering and removal of ether under reduced pressure gave a heavy yellow oil (crude yield: 0.26 g, 100%) which crystallized upon standing. Recrystallization from cyclohexane/hexane (10 ml) gave yellow crystals of 2-deuterio-9-fluorenone: yield: 0.20 g (74.7%).

4-Bromo-9-Fluorenone

4-Bromo-9-fluorenone was synthesized by using the previously published procedure (Suzuki, *et al.*, 1958) to give greenish-yellow crystals (2.0 g, 75% yield).

4-Bromo-9-Fluorene

To a 50 ml roundbottom flask was added 4-bromo-9-fluorenone (0.68 g, 0.00261 mol), hydrazine hydrate (99-100%) (1.6 ml) and ethylene glycol (16 ml). The reaction mixture was refluxed for 5 hr, cooled, poured into 25 ml of water, and filtered to give 0.62 g, 97% yield of 4-bromo-9-fluorene.

4-Deuterio-9-Fluorene

4-Deuterio-9-fluorene was synthesized by using the procedure described above to synthesize 2-deuterio-9-fluorenone to give a greenish-yellow crystalline solid (0.04 g, 80% yield).

Mass Spectra

Mass spectra were run on a Hitachi-Perkin-Elmer RMU-6E mass spectrometer at 70 ev at 2-3 X 10^{-6} torr.

MASS SPECTRAL DATA

Compound 1a m/e (%): 181 (7.5), 180 (100.0), 153 (3.8), 152 (34.5), 151 (21.5), 150 (13.3), 149 (1.0), 127 (1.1), 126 (9.1), 125 (2.9), 113 (1.1), 102 (2.6), 101 (2.0), 100 (2.3), 99 (3.7), 98 (3.5), 90-1/2 (1.3), 90 (5.4), 87 (3.8), 86 (1.9), 77 (2.4), 76-1/2 (3.6), 76 (22.6), 75-1/2 (3.2), 75 (9.5), 74 (4.9), 63 (9.5), 62 (2.8).

Compound 1b m/e (%): 183 (1.2), 182 (13.5), 181 (100.0), 180 (13.5), 153 (4.6), 152 (35.9), 151 (21.3), 150 (14.3), 149 (1.3), 127 (1.1), 126 (8.9), 125 (3.1), 122 (1.2), 102 (3.3), 101 (2.2), 100 (1.8), 99 (3.9), 98 (3.1), 90-1/2 (6.1), 88 (1.1), 87 (3.9), 86 (2.3), 77 (2.6), 76-1/2 (3.6), 76 (21.8), 75-1/2 (3.1), 75 (6.8), 74 (4.7), 63 (7.4), 62 (2.1).

Compound 1c m/e (%): 183 (1.4), 182 (17.9), 181 (100.0), 180 (10.8), 154 (6.0), 153 (35.2), 152 (23.3), 151 (14.2), 150 (3.5), 127 (5.7), 126 (5.0), 125 (1.2), 103 (1.4), 102 (1.1), 101 (1.0), 100 (1.4), 99 (1.6), 98 (1.1), 90-1/2 (3.2), 87 (1.5), 77 (3.3), 76-1/2 (10.1), 76 (4.3), 75-1/2 (1.4), 75 (3.0), 74 (1.9), 63-1/2 (1.8), 63 (1.9).

Compound 1d m/e (%): 183 (2.5), 182 (27.8), 181 (100.0), 180 (21.3), 153 (35.0), 152 (23.6), 151 (15.7), 127 (6.2), 126 (4.2), 125 (1.3), 103 (1.8), 102 (1.6), 101 (1.3), 100 (1.6), 99 (2.1), 98 (1.7), 90-1/2 (5.54), 88 (1.2), 87 (1.9), 86 (1.2), 78 (1.4), 77 (5.7), 76-1/2 (19.9), 76 (8.3), 75-1/2 (2.3), 75 (5.1), 74 (3.1), 64 (1.6), 63-1/2 (4.2), 63 (4.2), 62 (1.2).

Compound 2a m/e (%): 167 (12.4), 166 (100.0), 165 (77.5), 164 (7.9), 163 (12.4), 162 (1.1), 139 (5.8), 137 (1.1), 115 (2.5), 113 (1.2), 89 (1.2), 87 (1.3), 83-1/2 (1.3), 83 (11.9), 82-1/2 (13.8), 82 (7.8), 81-1/2 (3.5), 81 (1.5), 69-1/2 (3.3), 63 (2.0), 59 (1.5).

Compound 2b m/e (%): 168 (14.4), 167 (100.0), 166 (90.4), 165 (19.5), 164 (16.5), 163 (3.3), 141 (1.7), 140 (5.7), 139 (1.7), 138 (1.8), 116 (3.5), 115 (2.2), 114 (1.6), 113 (1.0), 111 (2.2), 98 (1.6), 97 (1.6), 95 (1.2), 91 (1.2), 90 (1.1), 89 (1.5), 88 (2.0), 87 (2.2), 84 (2.0), 83-1/2 (13.6), 83 (16.4), 82-1/2 (11.8),

82 (6.9), 81-1/2 (3.1), 81 (1.2), 79 (1.1), 77 (1.2), 76 (1.2), 75 (2.4), 74 (1.0), 71 (2.4), 70 (4.3), 69-1/2 (1.6), 69 (1.8), 67 (1.0), 64 (1.4), 63 (1.8), 60 (1.6), 59 (14.3).

Compound 2c m/e (%): 171 (1.1), 170 (12.1), 169 (100.0), 168 (76.9), 167 (33.0), 166 (9.7), 165 (6.8), 164 (2.9), 142 (1.8), 141 (3.6), 140 (1.7), 118 (1.0), 117 (1.7), 116 (1.0), 85 (1.6), 84-1/2 (9.4), 84 (8.9), 83-1/2 (7.6), 83 (5.4), 82-1/2 (2.4), 82 (1.4), 71 (1.1), 70-1/2 (1.6).

Compound 2d m/e (%): 171 (1.2), 170 (14.8), 169 (100.0), 168 (81.0), 167 (35.9), 166 (12.4), 165 (9.3), 164 (4.2), 163 (1.5), 142 (2.4), 141 (3.4), 140 (2.3), 139 (1.0), 118 (1.4), 117 (2.2), 116 (1.3), 90 (1.2), 89 (1.1), 88 (1.2), 87 (1.1), 85 (2.0), 84-1/2 (12.6), 84 (13.1), 83-1/2 (10.9), 83 (7.2), 82-1/2 (4.2), 82 (2.0), 75 (1.0), 71 (2.0), 70-1/2 (3.0), 70 (1.5), 64 (1.6), 63 (1.4).

ACKNOWLEDGEMENTS

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INTRACOASTAL TIDES OF UPPER LAGUNA MADRE, TEXAS[1]

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ABSTRACT

A 1-yr record of hourly water level measurements from Upper Laguna Madre, Texas, indicates water level variations occur primarily in response to estuarine-shelf exchanges in 3 forms. A dominant, semi-annual rise and fall of sea level is driven externally by thermohaline and dynamic processes in the Gulf of Mexico. Meteorologically forced variations in coastal sea level produce exchanges and thus intracoastal water level variations over time scales on the order of 1 to 2 weeks. Astronomical tides are relatively small perturbations superimposed onto the longer-period processes.

Water level data from Port Aransas, Texas, and Upper Laguna Madre are used to trace the movement and damping of tidal motions from the coast, through Corpus Christi Bay to the study site. Tidal crests move at approximately 4-5 km/hr, and the constituents are decreased to less than 25% of their amplitudes at the coast. Tidal motions in Upper Laguna Madre are primarily at diurnal periods, and K_1 and O_1 constituent amplitudes are both on the order of 2-3 cm. Comparison of tidal and non-tidal period water level variations indicates that tidal motions account for approximately 5% of the total observed variation in water level at this location.

INTRODUCTION

Laguna Madre, extending nearly 200 km along the South Texas Gulf coast, is a shallow, semi-enclosed coastal lagoon lying inside Padre Island. The width is generally between 2 and 3 km, and water depths are characteristically on the order of 1 m. Climatologically, the area is one in which the annual evaporation exceeds the precipitation, yet at times rainfall and freshwater run-off can be substantial. Partly for this reason, Laguna Madre has been the subject of considerable interest for many years. The resident plant and animal populations must be capable of tolerating a wide range of salinities between midsummer, when evaporation increases salinities to 60 parts/1000 or more (Behrens, 1966), and the fall months, when the annual precipitation curve is at a maximum and water may become decidedly brackish. Similarly, temperatures range annually from near freezing to as high as 40C in the shallow flats. In spite of these harsh environmental conditions, fish populations are abundant and the waters are heavily fished by sportsmen and commercial fishermen. Laguna Madre supports adult populations

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of fish the year around, in addition to serving as a nursery area for juvenile forms of fish and invertebrates.

The scientific interest and commercial value of the waters of Laguna Madre have stimulated studies over the past 30 years. Most of the published accounts deal with the biology or general ecology of the region. Hedgpeth (1947) included remarks dealing with the hydrography of Laguna Madre in a general study of the area. Some evidence for a wind-induced flushing of Laguna Madre waters is presented using salinity as a natural tracer. One phase of a 3-part ecological survey conducted by the Texas Game and Fish Commission was carried out in Laguna Madre north of the Land Cut (Simmons, 1957). This report is of a general nature and includes information relating to the local circulation and tides. Simmons notes that wind directions and water levels are interrelated, but no quantitative data are presented. Copeland, *et al.,* (1968) noted a similar relationship between winds and water levels in 3 short studies conducted in 1963.

The only purely hydrographic study of the area was that conducted by Collier and Hedgpeth (1950), which covered Texas intracoastal waters between Baffin Bay and Espiritu Santo Bay. A separate section was devoted to Laguna Madre, but the data presented were collected before the construction of the Intracoastal Waterway in 1949, and thus are largely of historical interest.

These previous studies provide a background for a more specific investigation of water level variations in Upper Laguna Madre. It has been shown qualitatively that tidal variations in water levels are small and often dominated by wind effects, but little quantitative information is available relating to tidal motions or the relative importance of tidal and long-period, nontidal processes. The purpose of this paper is to describe quantitatively the tidal and longer period water level variations, and thus to extend the results of previous studies of physical processes in Upper Laguna Madre.

THE OBSERVATIONS

Water level records were obtained from U.S. Army Corps of Engineers recorders located along the South Jetty of the Aransas Pass (Fig. 1) at Port Aransas, Texas, (27°50.25′N, 97°03.00′W), and at Marker 21 of the Gulf Intracoastal Waterway in Upper Laguna Madre (27°37.10′N, 97°14.32′W).

A 365-day time series of hourly observations over the period February 1, 1974 through January 31, 1975, provided the primary data base from which tidal and longer period water level variations were investigated. An overlapping 148.5-day water level record from the South Jetty in Port Aransas provided information on how tidal and longer period water level variations move into the study site from the northwestern Gulf of Mexico.

Water levels were recorded in analog form and read to the nearest 0.01 ft (3 mm) at hourly intervals relative to a datum plane 1 ft below mean sea level. High frequency noise in the water level records from both locations was effectively damped by the stilling well, and it is felt that values have a precision of approximately ±6 mm. Water levels were converted to cm above the datum before being analyzed.

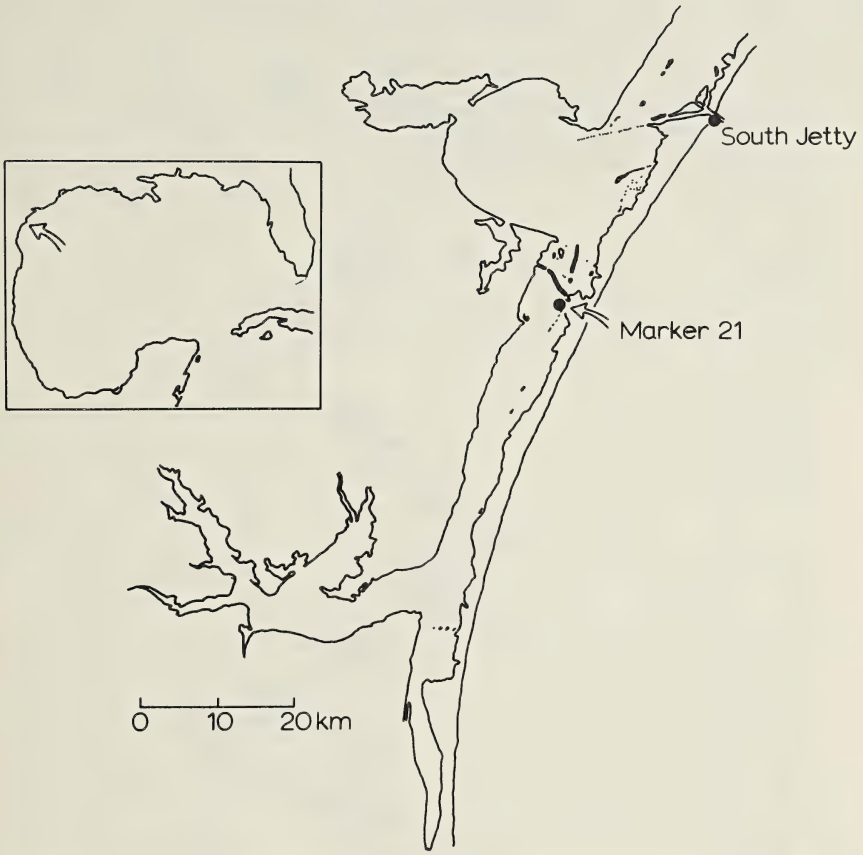

Figure 1. Locations of Marker 21 in Upper Laguna Madre, Texas, and the South Jetty at Port Aransas. Insert shows the study site in the northwestern Gulf of Mexico.

RESULTS

To put both tidal and longer period water level variations in Upper Laguna Madre in perspective, hourly values were plotted for the entire 365-day study period (Fig. 2). The results show largely in a qualitatively way the relative importance of water level variations occurring over time scales from a few hours to several months. Dominating the pattern in Fig. 2 is the very long period, semi-annual rise and fall of water level in the lagoon. High water occurs in late May and late October, while the semi-annual lows are found in late February and late July. The rise and fall in water level associated with these seasonal fluctuations is on the order of 50 cm.

Superimposed onto the very gradual rise and fall in sea level in Upper Laguna Madre are water level variations occurring over time scales on the order of 1 to 2

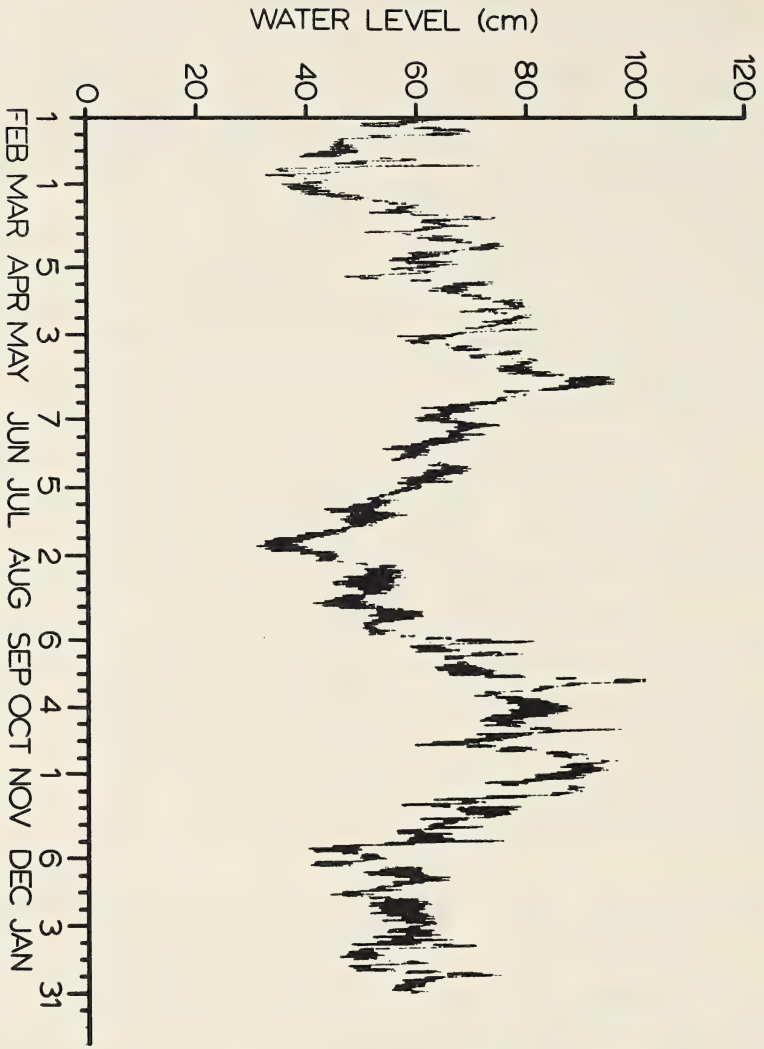

Figure 2. Time plot of hourly water levels, in cm, relative to a datum 1 ft below mean sea level, from Marker 21, February 1, 1974 through January 31, 1975.

weeks. The rise and fall of water level over these time intervals is quite variable, but generally on the order of 10-20 cm.

Diurnal and perhaps semi-diurnal astronomical tides appear as little more than high frequency and relatively insignificant noise on the longer period variations. There is some indication of a cycling in and out of phase of tidal constituents, significantly altering the relative importance of tidal period water level variations and those occurring over longer time scales.

The relative importance of water level variations occurring over a wide range of time scales was investigated quantitatively by computing an energy density spectrum from the year-long water level record. Fig. 3 is an energy density spectrum computed from 2-hourly water levels from Marker 21 recorded between February 1, 1974 and January 31, 1975. The computer program used for the calculations (Fee, 1969) involves a fast-Fourier transform technique after the linear trend has been removed from the data.

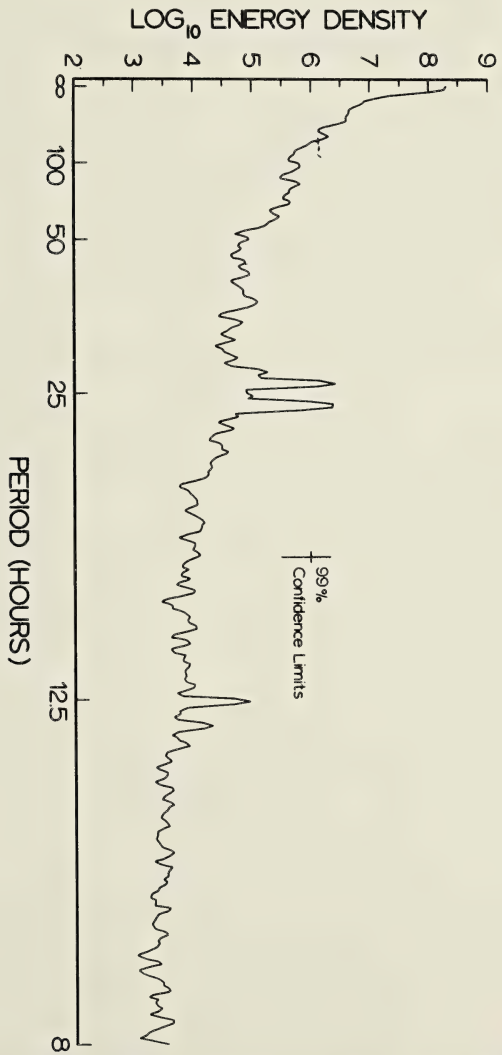

Figure 3. Relative energy density spectrum of 2-hourly water levels from Marker 21, February 1, 1974 through January 31, 1975. Vertical axis is in cm^2/cph. Spectral resolution is 0.000385 cph.

Fig. 3 shows a rather uniform decrease in energy density levels with decreasing period. The exponential decrease is interrupted first by the spectral peaks occurring at the O_1 and K_1 tidal constituent periods. Energy density values rise nearly 2 orders of magnitude above surrounding levels in that part of the spectrum. The M_2 and S_2 semi-diurnal constituents produce spectral peaks of lesser importance at periods of approximately 12.4 and 12.0 hrs. The spectrum has been plotted only to a period of 8 hrs, as no statistically significant spectral peaks were computed at shorter periodicities.

To investigate the possibility that water level variations observed in Upper Laguna Madre move in from the northwestern Gulf of Mexico through the Aransas Pass at Port Aransas (Fig. 1), a coherence-squared spectrum was computed from water level records obtained simultaneously over a 148.5-day period from the South Jetty at Port Aransas and Marker 21 in Upper Laguna Madre. Results are shown in Fig. 4. Three significant features stand out in the spectrum. Highest coherence-squared values occur at periods longer than about 50 hrs, resulting from long-period variations in meteorological, thermohaline and dynamic forces. These forces drive water against or away from the coast at Port Aransas, forcing a slow filling or draining of the intracoastal bays. The estuarine-shelf exchanges eventually affect the study site, approximately 35 km from the coast along the Corpus Christi Ship Channel and through Corpus Christi Bay.

Coherence-squared values drop off abruptly and remain low between periods of approximately 50 and 30 hrs, then rise sharply at the diurnal tidal periodicities. Both the O_1 and K_1 tidal constituents can be identified in the spectrum by slightly higher coherence-squared values.

The semi-diurnal motions are statistically significant at the M_2 period only, with a coherence-squared value of over 0.65 at a period of 12.5 hrs. The immediate decrease in coherence suggests that the S_2 constituent does not move in from the Gulf to the study site. The spectral peak in Fig. 3 may therefore be a result of local wind effects.

Table 1 gives the phase lag of water level variations at diurnal and semi-diurnal periods recorded at Marker 21 relative to those recorded at the coast at Port Aransas. At the diurnal periods, between 23.3 and 27.0 hrs, phase lags of between 122° and 132° are computed. These are equivalent to time lags of between 8.5 and 9.3 hrs. With a distance of approximately 35 km from the coast to the study site, this corresponds to a speed of propagation of the tidal wave crest of about 4 km/hr. At semi-diurnal periods of between 12.3 and 12.7 hrs, the phase lag is between 204° and 206°, corresponding to a time lag of between 7.0 and 7.2 hrs and a speed of propagation of approximately 5 km/hr.

Twenty-nine day blocks of hourly water levels were used to compute the harmonic constants of the principal tidal constituents with a computer program described by Dennis and Long (1971). Tidal constituent amplitudes, in cm, and local phase angles are shown on harmonic dials for the K_1 and O_1 partial tides in Fig. 5. Points for the O_1 tidal constituent are fairly tightly clustered, with phase angles

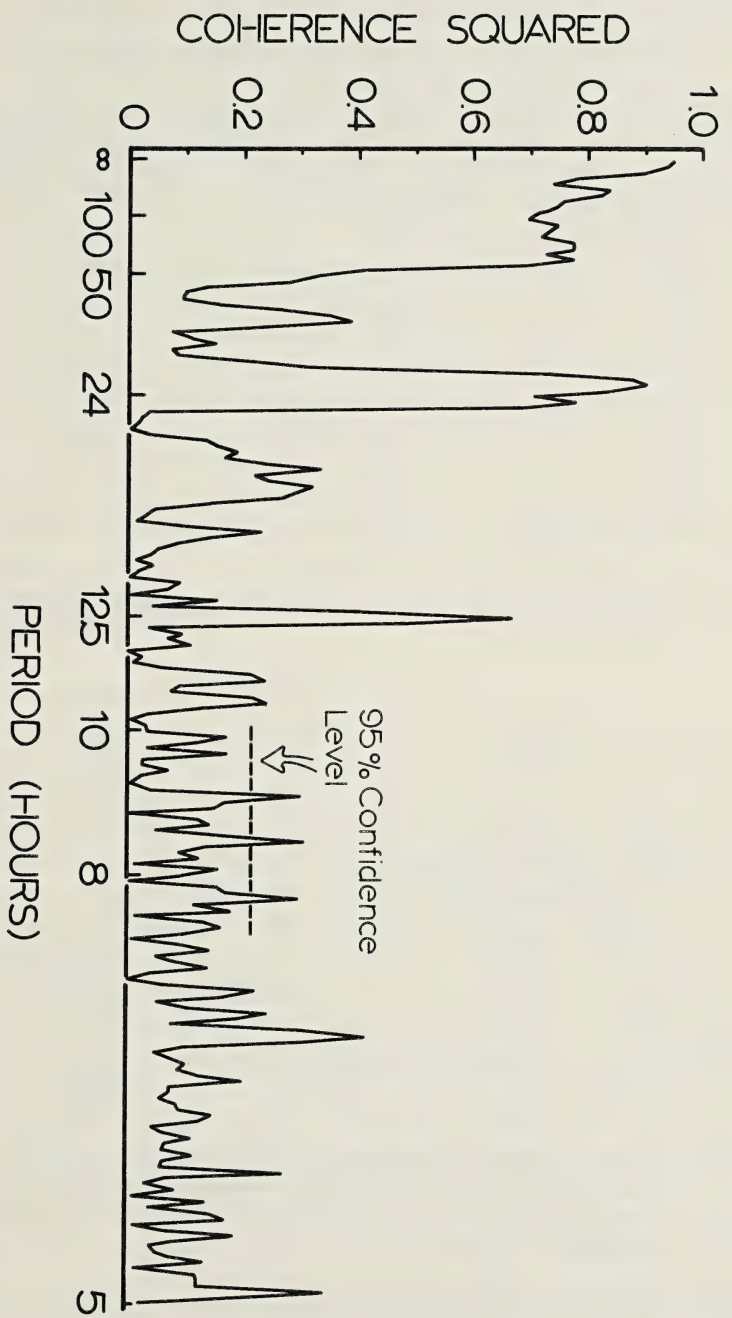

Figure 4. Coherence-squared spectrum for South Jetty and Marker 21 water level data, January 30 through June 27, 1974. Spectral resolution is 0.0001 cph.

TABLE 1

Phase Lags of Diurnal and Semi-diurnal Period Water Level Variations at Marker 21, Relative to Those at Port Aransas. January 30 to June 27, 1974.

Period (Hrs)	Phase Lag at Marker 21 (Degrees)	Corresponding Time Lag (Hrs)
27.0	124	9.3
26.3	125	9.1
25.6	125	8.8
25.0	122	8.5
24.4	125	8.5
23.8	130	8.6
23.3	132	8.5
12.7	204	7.2
12.5	205	7.2
12.3	206	7.0

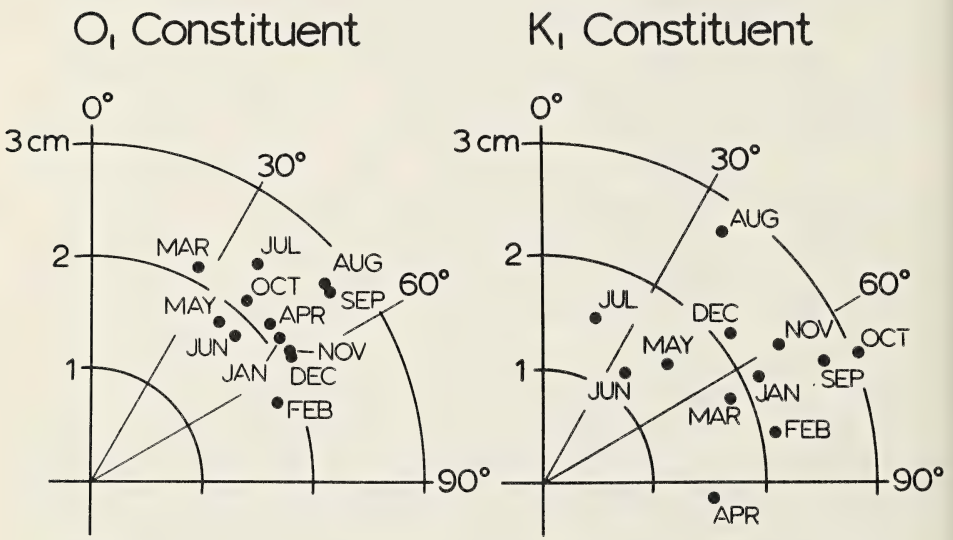

Figure 5. Harmonic dials of amplitudes, in cm, and local phase angles of the O_1 and K_1 partial tides for Marker 21, February, 1974 through January, 1975. Harmonic constants computed from 29-day records, starting on the first day of the indicated month.

generally between 30° and 60°, and amplitudes between 1.8 and 2.8 cm. For comparison, O_1 constituent amplitudes computed from South Jetty water level data are approximately 12 cm (Smith, 1974). There is no well-defined seasonal

variation in amplitude, however, phase angles computed for the fall and winter months are somewhat larger than those computed for the spring and summer months.

The harmonic dial for the K_1 constituent shows a pattern that is similar though somewhat more spread out. Amplitudes are between about 1 and 3 cm, and local phase angles fall between approximately 30° and 90°. There is some indication that higher amplitudes are characteristic of the fall and winter months. K_1 constituent amplitudes at Port Aransas are approximately 12 cm (Smith, 1974), suggesting that diurnal period tidal constituent amplitudes at the study site have been damped to less than 25% of their amplitudes at the coast.

The relative importance of tidal and non-tidal water level variations at Marker 21 was investigated by numerically filtering the 365-day water level record to remove tidal periodicities. The "D39" Doodson-Warburg type filter described by Groves (1955) was chosen for this part of the analysis. The variance of the water level data was computed both before and after filtering. Ninety-five percent of the total variance remained in the filtered water level data, indicating that tidal-period processes contribute a small fraction of the variation in water level at this location. In contrast to this, variance in water level records from the South Jetty at Port Aransas appears to be approximately equally distributed between tidal and nontidal periodicities.

DISCUSSION

Aside from set-up and set-down effects produced locally by surface windstress, intracoastal water level variations occur primarily in response to the estuarine-shelf exchanges driven by variations in coastal water levels. The high coherence-squared values in Fig. 4, along with the computed phase angles, clearly demonstrate that both tidal and the longer period water level variations recorded at Marker 21 in Upper Laguna Madre appear first at the South Jetty at Port Aransas. Fig. 4 also shows that highest coherences are found in the very long period part of the spectrum. Thus, it is not surprising that the dominant feature of water level variations in Upper Laguna Madre is the semi-annual rise and fall of sea level characteristic of coastal locations along the northwestern rim of the Gulf of Mexico.

The causes of the semi-annual variations in coastal sea level have been suggested by Marmer (1954), Whitaker (1971), and Sturges and Blaha (1976), and appear to include both thermohaline and dynamic forcing. The spring run-off is primarily responsible for the relatively high coastal water levels in May. Seasonal warming and cooling produce a maximum expansion and contraction of shelf waters in September-October and January-February, respectively. Sturges and Blaha (1976) have recently suggested that the July minimum may be related to temporary storage of water in a large anticyclonic gyre in the western Gulf of Mexico maintained by the curl of the windstress. It is apparent from Fig. 2 that these sometimes distant processes, acting on the waters of the Texas shelf and in the waters

of the open Gulf, have a profound and dominant, if indirect effect on water levels in Upper Laguna Madre.

Similarly, the water level variations occurring in Upper Laguna Madre over time scales on the order of 1 to 2 weeks can be attributed to the slow exchange of water between intracoastal areas and the inner continental shelf in response to meteorological forcing. Smith (1977) has shown statistically significant relationships exist between variations in the regional pressure field over the central Texas shelf and variations in the volume of Corpus Christi Bay. Results suggest that bay-shelf exchanges are driven both by the cross-shelf component of the wind, producing a set-up and set-down of coastal water levels over time scales on the order of 4-6 days, and by the longshore component of the wind, producing a cross-shelf Ekman transport in the surface layer over longer time scales. Again, these estuarine-shelf exchanges have a significant local effect on water levels recorded in Upper Laguna Madre. The approximately weekly or biweekly addition or removal of volumes of water sufficient to raise or lower the water level by 5-10 cm must constitute an effective flushing mechanism.

The sympathetic tidal motions in Upper Laguna Madre provide a relatively small but nevertheless dependable mechanism for exchanging water with Corpus Christi Bay and eventually with the Gulf of Mexico. The low amplitudes of the individual tidal constituents in the study area (Fig. 5) are a direct result of the 35 km of shallow bays and constricting channels through which tidal motions must propagate. All of the principal tidal constituents' amplitudes are less than approximately 3 cm. While individual tidal constituents are geometrically additive, they periodically cycle in and out of phase, and one must still think of tides at the study site as being on the order of a few centimeters. Price (1971) has stated that the range of the daily astronomical tide in the northern Laguna Madre is 1.0 ft, but this appears to be about an order of magnitude too high.

The numerical filtering of the water level data to remove tidal-period variations demonstrates clearly that non-tidal forcing is of primary importance in Upper Laguna Madre. These aperiodic variations are as unpredictable as the meteorological events that cause them, however, they must be considered in any discussion of water level variations in this area.

ACKNOWLEDGEMENTS

The author would like to express his appreciation to Mr. D. T. Graham, Chief of the Engineering Division of the Army Corps of Engineers in Galveston, for providing water level data used in the study, and to Mr. William MacNaughton and Mr. Jim Evans for their help in the reduction and analysis of the data. Financial support for this study was provided by the National Park Service, under Contract CX 7000040146.

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Notes Section

REPRODUCTIVE SEASONS IN *GAMBUSIA AFFINIS* AND *GAMBUSIA GEISERI* (OSTEICHTHYES:POECILIIDAE) FROM SOUTHCENTRAL TEXAS—

Annual reproductive cycles were observed in the viviparous mosquitofishes *Gambusia affinis* and *G. geiseri* from the San Marcos, Texas, area from March 1974, to February 1975. The results are reported herein, since no previous data are available from southcentral Texas.

Gambusia affinis were collected with a long-handled dipnet each month from the following habitats (annual surface water temperature ranges given in parentheses): (1) a shallow pond on the Southwest Texas State University (SWTSU) campus (13-32 C), (2) a shallow, stagnant slough arm of Spring Lake, the impounded origin of the San Marcos River (15-29 C), (3) the shallow Blanco River below Five Mile Dam, 5 mi north of San Marcos (16-33 C), and (4) the springfed, thermally consistent San Marcos River headwaters (20-22.5 C). *Gambusia geiseri* were taken quarterly from the headwaters of the San Marcos River. At least 11 females (usually 12 or 13) from each sample were dissected and the presence of eyed embryos or later developmental stages was used as the criterion for gravidity.

Gambusia affinis was reproductive from February through October in the Blanco and San Marcos Rivers, and from February through December in Spring Lake and the SWTSU pond (Table 1). Annual reproductive cycles reported for *G. affinis* from other localities include: (1) April through October, Illinois (Krumholz, L.A., 1948, *Ecol. Monogr.*, 18:1); (2) March through October, Granite Co., Montana (Brown, C.J.D., and A.C. Fox, 1966, *Copeia*, 1966:614); and (3) March through September, Menard Co., Texas (Hubbs, C., 1971, *Bull. Tex. Mem. Mus.*, 19:1). Therefore, it seems that the reproductive season of *G. affinis*

TABLE 1

Incidence of Gravidity in *Gambusia affinis* and *Gambusia geiseri*.

Species	Habitat	No. Gravid Females/No. Females Examined/Month J	F	M	A	M	J	J	A	S	O	N	D
Gambusia affinis	SWTSU pond	0/12	0/12	6/17	10/13	7/13	5/13	3/12	4/13	5/12	2/14	0/12	1/12
	Spring Lake slough	0/12	0/12	5/13	11/14	10/16	6/13	8/13	6/12	3/12	4/13	0/13	1/11
	Blanco River	0/18	0/20	1/16	9/18	6/16	8/14	5/13	7/23	6/13	0/13	0/15	0/12
	San Marcos River	0/18	0/15	10/14	9/12	6/18	6/17	6/14	3/15	6/13	0/13	0/17	0/13
Gambusia geiseri	San Marcos River		6/18			7/14			8/14			7/12	
		Monthly Totals, Four Stations Pooled											
Gambusia affinis													
No. Gravid Females/ No. Females Examined		0/60	0/59	22/60	39/57	29/63	25/57	22/52	20/63	20/50	6/53	0/57	2/48
Percent Females Gravid		0.0	0.0	36.7	68.4	46.0	43.9	42.3	31.7	40.0	11.3	0.0	4.2
Mean Std. Lgth. of Gravid Females (mm)		–	–	31.2	34.9	33.0	31.7	29.8	29.9	31.5	34.0	–	37.5

in southcentral Texas is slightly longer than in more northern latitudes, perhaps due to the warmer southern climate. However, the relative similarity of reproductive seasons in *G. affinis* from widely separated latitudes (Illinois, Montana, and Texas) supports Hubbs' (1971, *Bull. Tex. Mem. Mus.*, 19:1) theory that length of the photoperiod rather than temperature is the primary control of the reproductive season in *G. affinis*. Additional observations in this study further support that theory since length of the reproductive season in *G. affinis* from the thermally consistent San Marcos River appears to be relatively similar to that in *G. affinis* from thermally fluctuating environments (Table 1).

Fertilization of mature ova began in the overwintered parent stock in mid-February, as eyed embryos were present during the first week of March. Since the average gestation period in *G. affinis* is about 24 days (Krumholz, L.A., 1948, *Ecol. Monogr.*, 18:1), the season's first young were probably born around mid-March.

Reproduction reached a peak in April, when a portion of the parent stock were gravid with their first brood of the season, and another portion which gave birth in March were gravid with their second brood. The 30-day brood interval reported for *G. affinis* during the spring months (Carlson, D.R., 1969, *Tex. J. Sci.*, 21:167) supports this observation.

Gambusia affinis mature sexually at 4 to 6 weeks of age and the average gestation period is 24 days (Krumholz, L.A., 1948, *Ecol. Monogr.*, 18:1). Therefore, eyed embryos first occurred in young-of-the-year about the first of May. The emergence of this season's first brood into the reproductively active portion of the population in May was evident by the observed decrease in the average standard length of gravid females from April to May (Table 1). Also, large numbers of sexually immature females were incorporated into the population at this time, causing a decrease in the overall incidence of gravidity in females from April to May.

The decrease in gravidity incidence and average size of gravid females each month from May through August was probably due to (1) the continual influx of immature young into the population, (2) the increasing contribution of the season's young to the reproductively active population, and (3) death of the parent stock. This is a dynamically complex period, as the parent stock continue to bear young until they disappear from the population during July and August (Krumholz, L.A., 1948, *Ecol. Monogr.*, 18:1), and as this season's offspring mature and become reproductively active.

A second reproductive peak occurred in September, during maximal concurrency of reproductive activity by all of the season's broods born from March through July. Fish born after mid-July don't reproduce that year, but rather overwinter and are present at the beginning of the following season as the parent stock (Krumholz, L.A., 1948, *Ecol. Monogr.*, 18:1). Since post-July offspring were absent from the reproductively active portion of the population, the average length of gravid females continuously increased from July through December (Table 1) as growing young born in spring and early summer were the sole contributors to reproduction during this period.

Krumholz (1948, *Ecol. Monogr.*, 18:1) reported that the portion of the season's young which were reproductively active, i.e., the young born between March and mid-July, do not survive the winter. The decreasing overall incidence of eyed embryos after September was probably due to the cumulative disappearance of those fish from the population, leaving a larger percentage of immature fish. A few females, most likely the last of the sexually mature, mid-summer's offspring, gave birth in December (Table 1).

Gambusia geiseri appears to reproduce year around in the headwaters of the San Marcos River, as reproductive activity peaked in November and 1/3 of the February females carried eyed embryos (Table 1). This is in contrast to *G. affinis* from the San Marcos River, which exhibited no reproduction from October through January.

Gambusia geiseri, considered endemic to the constant temperature headwater regions of the San Marcos and Comal rivers (Hubbs, C., and V.G. Springer, 1957, *Tex. J. Sci.*, 9:279),

has apparently evolved to take maximal advantage of its thermally stable environment by extending its breeding season year around. This adaptation may be an important factor in the observed ability of *G. geiseri* to predominate over the sympatric *G. affinis* in the upper San Marcos River.

Appreciation is extended to Mr. John R. Schenck for his critical review of the manuscript. —*Jack R. Davis, Texas Water Quality Board, Pecos 79772.*

A NEW METHOD FOR THE PURIFICATION OF VINYLTRIMETHYLSILANE—We have observed that the purification of vinyltrimethylsilane (B.P. = 54.6 C) prepared in this laboratory is somewhat tedious because of the difficulty of separating it from tetrahydrofuran (B.P. = 67 C) which is required in the Grignard reaction route to vinylsilanes (S.D. Rosenberg, J.J. Walburn, T. Stankovich, A.E. Balent and H.E. Ramsden, 1957, *J. Org. Chem.*, 22:1200). Furthermore, commercially supplied samples of vinyltrimethylsilane have also been found, at times, to be contaminated with impurities boiling very close to the title compound (e.g., acetone, B.P. = 56 C was observed in one commercially supplied sample). We herein report a purification scheme for vinyltrimethylsilane which is based on the formation and subsequent decomposition of its CuCl complex (J.W. Fitch, D.P. Flores, and J.E. George, 1971, *J. Organometal. Chem.*, 29:263). Using this scheme a commercial sample of vinyltrimethylsilane (86% pure by G.C.) was purified in one step (99% pure by G.C. in 54% yield). Similarly, a mixture containing 10% THF was converted in one step to 98.4% pure vinyltrimethylsilane in 62% yield. Over 98% of the starting material was accounted for in each case.

Experimental Procedure

A Schlenk tube (100 ml, Tube A) was loosely packed with glasswool and anhydrous CuCl (3.5 g, 0.035 mmole) (prepared according to W.J. Jolly, 1960, *Synthetic Inorganic Chemistry,* Prentice-Hall N.J., p. 142). A second empty Schlenk tube (100 ml, Tube B) was attached to the first by means of vacuum tubing, and both tubes were first evacuated and then filled with argon. Tube B was then chilled to −78 C (Dry Ice and ethanol), and impure vinyltrimethylsilane (6.5 ml, 4.5 g) was added to it against a counter current of argon. The entire system was then evacuated, and the cold trap was removed from tube B and placed on tube A. As tube B warmed to room temperature the vinylsilane distilled into tube A and formed the CuCl complex. The cold trap was then put back onto tube B for 10 additional min after which it was removed and the entire system was allowed to equilibrate *in vacuo* at ambient temperature for 2 hrs. The system was once again filled with argon and tube B was emptied (it contained the impurities and most of the excess vinylsilane). At this time the complex in tube A was briefly dried by pumping on it for 1 min. Tube B was then replaced and cooled to −78 C; the entire system was once again evacuated, and tube A was heated at 45 C for 15 min to decompose the CuCl complex and distill the purified vinyltrimethylsilane into the cooled Schlenk tube B.

The financial support of the Robert A. Welch Foundation (Grant AI-306) is gratefully acknowledged.—*John W. Fitch and Michael Roesch, Department of Chemistry, Southwest Texas State University, San Marcos 78666.*

THERMAL ECOLOGICAL FEATURES OF *CYPRINODON ELEGANS* AND *GAMBUSIA NOBILIS*, ENDANGERED TEXAS FISHES—The Comanche Springs pupfish (*C. elegans*) and Pecos gambusia (*G. nobilis*) are endangered species that occur together in a heavily impacted system of desert springs and streams in Trans-Pecos, Texas (Hubbs and Echelle, 1972, *Symposium on Rare and Endangered Wildlife of the Southwestern United States*, New Mexico Dept. Game and Fish, Santa Fe, pp. 147-167). Nothing is known of their thermal ecology, although both species are cultured in artificial refugia at

Balmorhea State Park, Texas, and the U.S. Fish Hatchery, Dexter, New Mexico. We investigated their summer thermal preferences and maximum thermal tolerances to provide information for their management.

METHODS

Adults were collected September 30, 1972 in 21–25 C water ca 50–100 m below Phantom Cave Springs, Jeff Davis Co., Texas. Males of *C. elegans* were 47–52 mm, females 45–50 mm; males of *G. nobilis* 20–27 mm, females 37–42 mm (all standard length). Only 50 of each species were taken, because of their precarious status. These were kept at 20 C and LD 12:12 no less than 72 hrs before testing. In all experiments water temperatures were recorded next to individual fish with thermister probes and a multichannel electric thermometer. Individuals were used only once, and equal numbers of both sexes were used in each test.

Critical thermal maximum (CTMax) was measured as behavioral death (loss of righting response). Fish were placed individually in 200 ml of conditioned, aerated 20 C tap water in jars immersed in water baths at 0900–1100 (morning) and 1500–1700 (afternoon) hrs CST. Water temperature was elevated 0.5–1.0 C/min to prevent acclimation (at the risk of thermal shock) until loss of righting, after which fish were allowed to recover. All did.

For thermal preferenda, specimens were placed in a 15 x 140 cm glass trough containing 20 C, conditioned tap water at a depth of 3 cm. Positions of individuals (4 pupfish alone, 4 gambusias alone, or 4 of each species together in afternoon only) were recorded at 15 min intervals at 0730–0830 and 1300–1400 hrs while the water remained at 20 C. Then a 11–35 C thermal gradient was established with a thermostatic heater at one end of the trough and ice at the other. Positions of each fish were recorded every 15 min at 0900–1100 and 1500–1700 hrs.

RESULTS

Males and females of the pupfish did not differ greatly in size or CTMax by contrast with the gambusias. With 8 males and 8 females, respectively, the afternoon CTMax was 40.1 ± 0.09 and 40.5 ± 0.12 in the pupfish and 38.1 ± 0.15 and 39.3 ± 0.17 in the gambusias ($\bar{x}$ ± SE C). Only the difference in *G. nobilis* was significant ($t = 2.58$, $P < 0.05$) and in keeping with earlier evidence of higher thermal tolerances in female gambusias generally (Hagen, 1964, *Southwest. Natur.,* 9:6), although sexual differences in CTM for other pupfishes have not been reported.

Pupfish tested in the afternoon tolerated a significantly higher CTMax than those in the morning. Mean values for 4 males plus 4 females were 40.4 ± 0.11 and 39.6 ± 0.13 C respectively ($t = 3.40$, $P < 0.01$). The same test of *G. nobilis* did not yield a significant difference. Among other pupfishes only *C. bovinus* has been investigated adequately in this manner (Kennedy, 1977, *Copeia* 1977:93), and it too has a significantly higher CTMax in the afternoon.

Lacking a thermal gradient both *C. elegans* and *G. nobilis* tended to aggregate at the ends of the trough, regardless of the time of day. In the gradient each species alone showed a significant preference for 21–25 C water in the morning and 26–30 C water in the afternoon (Table 1; $X^2 = 13.7, 18.9$, $P < 0.01$ with 2 x 4 and 2 x 3 contingency tables). A change in acclimation may explain this difference. Yet when tested together *C. elegans* chose a significantly lower thermal position than its preferendum in isolation, while the modal position of *G. nobilis* did not shift ($X^2 = 20.8$, $P < 0.01$; vs. 3.8, $P > 0.05$, respectively with 2 x 4 and 2 x 3 contingency tables).

Phantom Cave Springs are usually 22–26 C (White, *et al.,* 1941, *U.S. Geol. Surv. Water Supply Paper,* 849C:83). On a typical afternoon (1200–1500 hrs, August 4, 1971) adult

pupfish were concentrated on and near the bottom of the concrete outflow canal in 25–27 C water, while gambusias remained at and near the surface in 27–29 C water. This substantiates our experimental evidence of thermal segregation, although it may relate primarily to aggression by the pupfish and fundamentally different spatial niches instead of different thermal preferenda.

TABLE 1

Number of *Cyprinodon elegans* and *Gambusia nobilis* per 5 C in a Laboratory Thermal Gradient (N = 4 of Each Species/15 min at 0900–1100 (AM) and 1500–1700 (PM Hours)

		Thermal Gradient (C)				
		11–15	16–20	21–25	26–30	31–35
C. elegans	AM	1	4	28	2	1
	PM		1	6	19	10
G. nobilis	AM	1	1	32	2	
	PM		1	13	22	
Species together in PM only						
C. elegans			6	27	2	1
G. nobilis			3	4	28	1

CONCLUSIONS

We believe that eurythermal water conditions should be designed to protect *C. elegans* and *G. nobilis* in an artificial refuge such as the concrete canal at Balmorhea State Park. The temperature range should approximate 20–30 C in August–September. This encompasses the morning and afternoon thermal preferenda of both species, does not approach their CTMax too closely, and permits habitat partitioning that may relate to thermal segregation and the bottom-dwelling versus surface positions of the pupfish and gambusia respectively.

ACKNOWLEDGEMENTS

We thank Anthony A. Echelle and J. Spencer Johnston for help with this study, supported by the senior author under tenure of a John Simon Guggenheim Memorial Fellowship 1970–71.*–Frederick R. Gehlbach, Christopher L. Bryan, and Harley A. Reno, Department of Biology and Institute of Environmental Studies, Baylor University, Waco 76703.*

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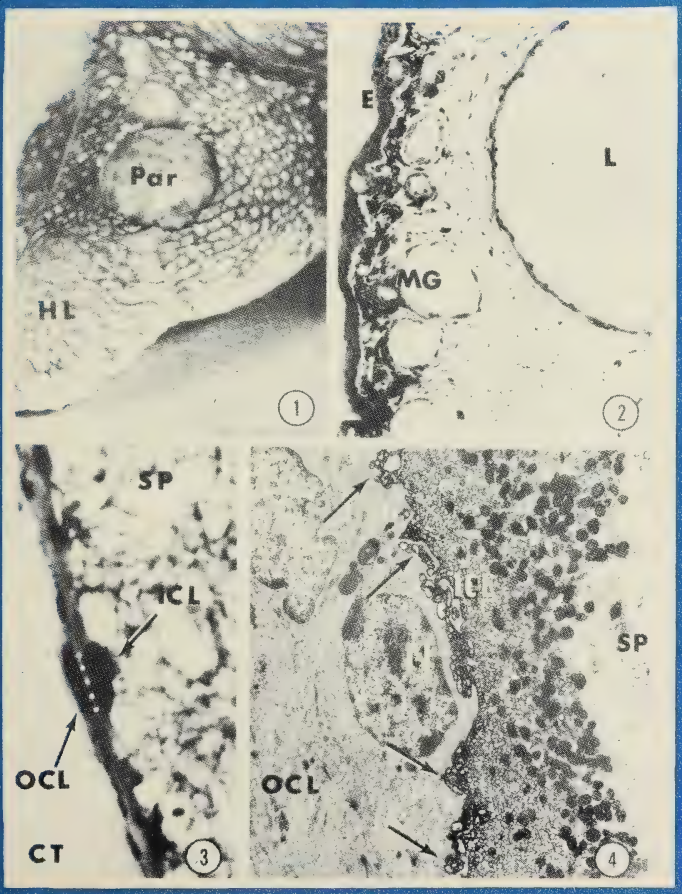

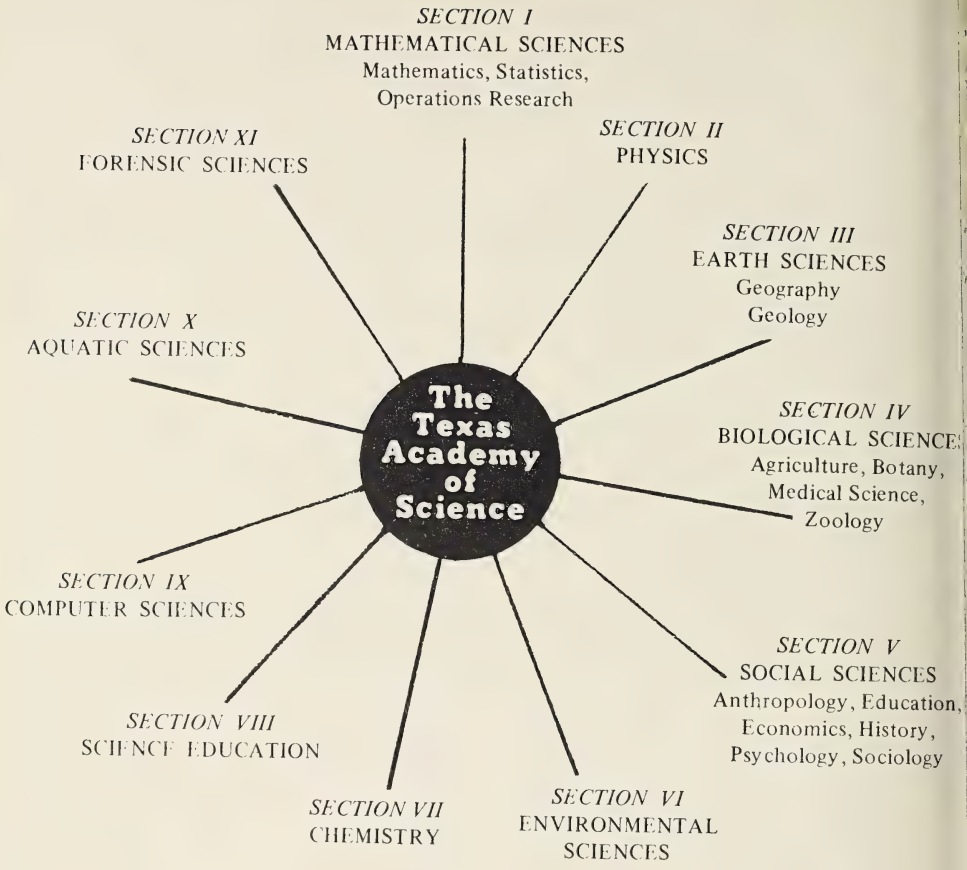

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CORRECTIONS

Volume XXVIII, Nos. 1–4, Table 1, Lines 13–14, should read:

	Tukey's Test					
Salinity (ppt)	0.0	2.0	4.0	6.0	9.0	13.0
Mean survival (%)	87.5	84.1	82.5	80.8	72.8	52.0

Volume XXIX, Nos. 3 and 4, Page 240, Line 30, "*Peromyscus inornatus*" should read "*Perognathus inornatus*" and Line 32, "*Peromyscus hispidus*" should read "*Perognathus hispidus.*"

PRECIPITATION CLIMATOLOGY FOR THE TEXAS HIGH PLAINS

by DONALD R. HARAGAN

Department of Geosciences,
Texas Tech University, Lubbock 79409

ABSTRACT

Advances in the science of weather modification have provided an opportunity for significant progress in the area of precipitation management. The problem of evaluating cloud-seeding projects has been accentuated, however, by a lack of adequate statistical data to define quantitatively the natural variability characteristics of the regions in question. The climatology presented here provides a base for proper evaluation. Quantitative assessment of the spatial and temporal variability of average (1944-73) precipitation is provided.

INTRODUCTION

The economic future of Northwest Texas is dependent to a large extent on the solution of hydrologic problems related to the occurrence of precipitation. The problem of providing an adequate water supply is paramount. Several projects for augmenting the natural water supply have been proposed including water importation, artificial groundwater recharge, and precipitation augmentation by cloud-seeding. Proper evaluation of these proposals, particularly the one involving weather modification, emphasizes the importance of a more comprehensive knowledge of the temporal and spatial variation of precipitation in the area. The decision processes involved in the evaluation of weather modification efforts are related ultimately to a knowledge of natural precipitation variability which defines the precipitation climatology for a particular area. Information of this type is also essential in solving a variety of problems dealing with irrigation, crop yields, drainage and overall water supply.

PERIOD OF STUDY

The initial task of selecting a suitable data base was important. Three objectives were established as follows:

1. to provide a representative data sample in time and space (30-yr period if possible).
2. to utilize only those data from National Weather Service stations or cooperative observers officially recognized by that agency.

Accepted for publication: June 13, 1977.

The Texas Journal of Science, Vol. XXX, No. 2, June, 1978.

3. to avoid, where possible, contamination from operational or experimental weather modification projects.

The period from 1944 to 1973 was selected as the time interval most closely satisfying the above criteria. Although an operational hail suppression project was active in a small portion of the area from 1970 to 1973, no evidence was found to support the hypothesis that artificial effects had influenced the isohyetal patterns in the target area. This conclusion was based on a detailed comparison between the 2 periods, 1944-1973 and 1944-1969. Thus, the 30-yr period from 1944 to 1973 was selected as the data base for the investigation.

TEMPORAL VARIATION OF PRECIPITATION

The region of Northwest Texas under investigation is shown by the hatched area in Fig. 1. The climate is continental, characterized by rapid changes in temperature, marked extremes and large temperature ranges both daily and annually.

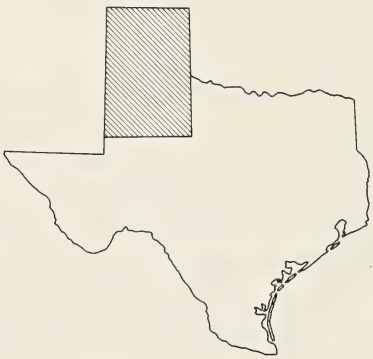
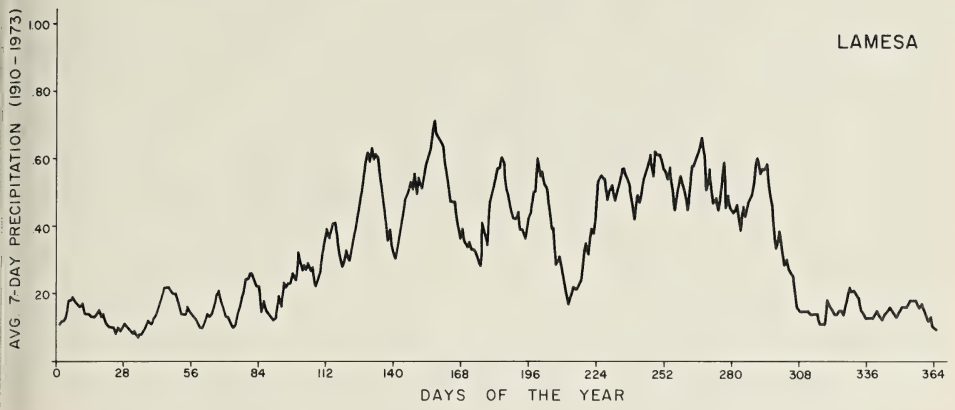


Figure 1. The region of Northwest Texas under investigation is shown by the hatched area.

Peak rainfall generally occurs in late spring and early summer. Approximately 43% of the average annual precipitation falls during the 3-month period, May through July, while 72% falls during the 6-month period, April through September (Haragan, 1976). The 5-month period, November through March, is a dry season as colder and drier polar air masses move south cutting off the flow of warm moist air from the Gulf of Mexico.

Fig. 2 shows the annual distribution of precipitation for 7 representative stations in the area. The curves represent running 7-day averages for the total period of record at each station. Records at Amarillo and Crosbyton are continuous since 1892 while the record at Plainview began in 1894. Records at Dalhart, Miami, Lamesa and Lubbock began in 1906, 1906, 1910 and 1912, respectively. Note that all stations reflect the spring and summer maximum previously described. Probably the greatest contrast is shown by a comparison of Miami and Lamesa. At Miami, the sharp peak in early June is followed by a rapid decrease


LAMESA
AVG 7-DAY PRECIPITATION (1910-1973)
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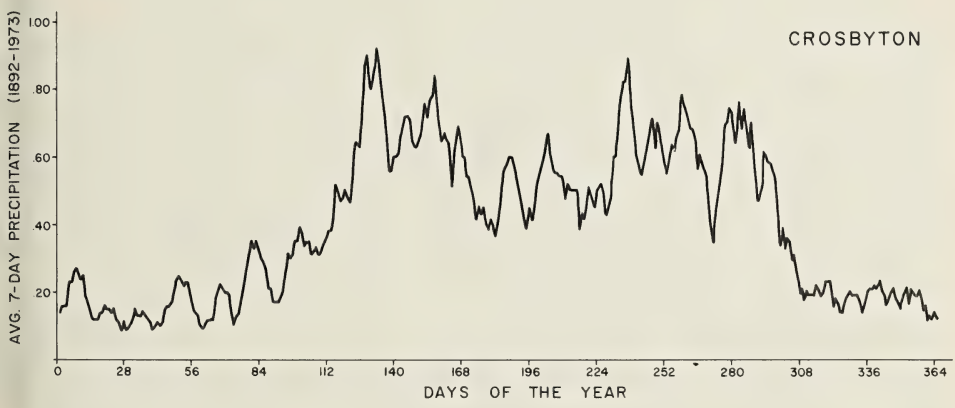
DAYS OF THE YEAR


CROSBYTON
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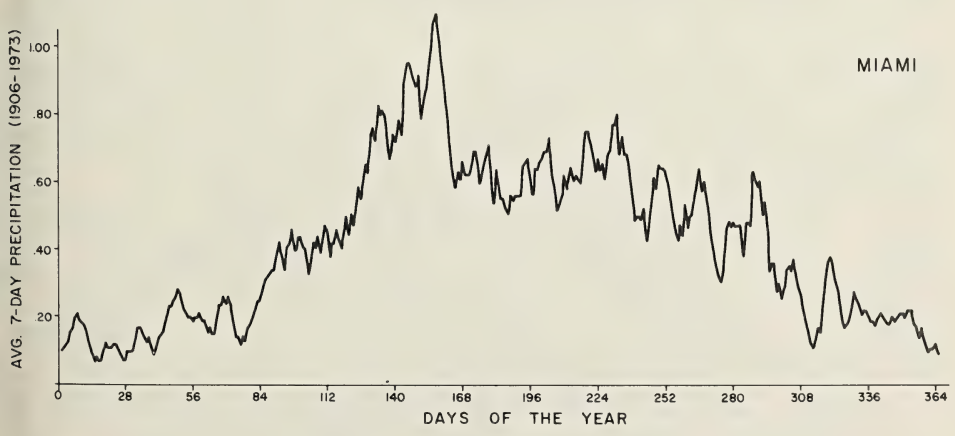
MIAMI
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(Figure 2)

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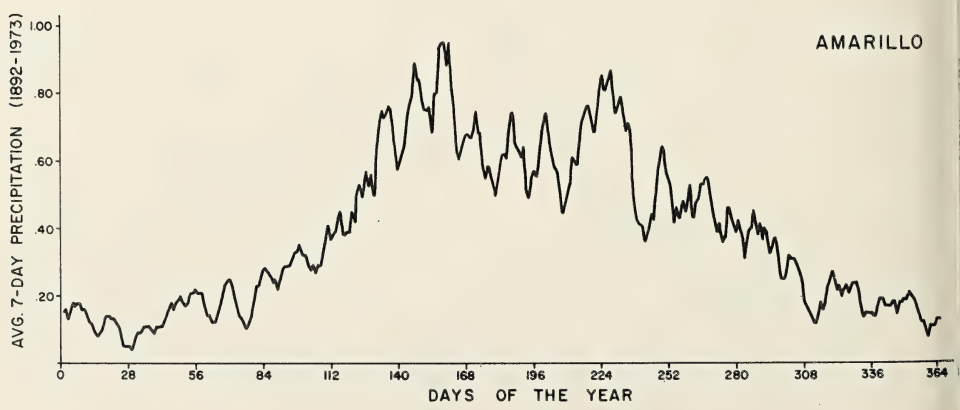

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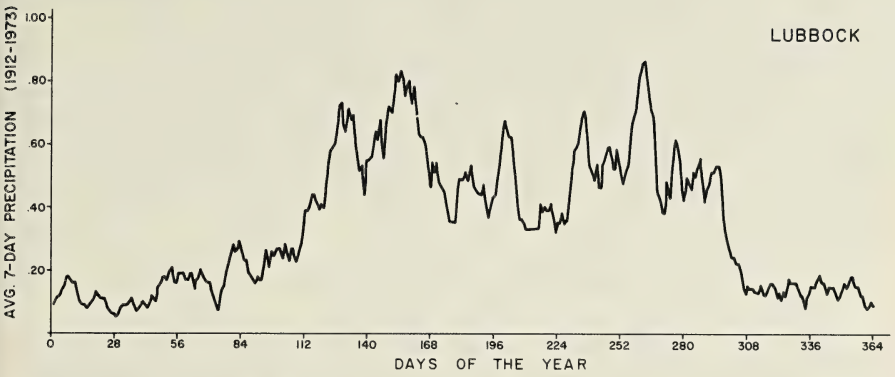


Figure 2. Annual distribution of precipitation for 7 representative stations in the study area.

and then a lesser but steady decrease to the end of the year. The curve at Lamesa, on the other hand, is much flatter, indicating a broad maximum extending throughout the summer and fall with the exception of a rather distinct minimum near August 1. Lubbock, Amarillo and Crosbyton tend to be more bi-modal with maxima occurring in the spring and again in the late summer or early fall. Several of the precipitation singularities, for example the peak at Lubbock in mid-September, are of interest and should be subjected to further investigation.

Another aspect of the temporal variation is illustrated by Fig. 3. This diagram represents annual precipitation averaged over space and time for the Texas Plains vicinity. Time-averaging involves 5-yr running means for the entire period of record. Space-averaging is over 9 stations: Amarillo, Crosbyton, Dalhart, Lamesa, Lubbock, Miami, Plainview, Post and Snyder. It is obvious from a cursory inspection of the figure that the natural variability of annual precipitation is large.

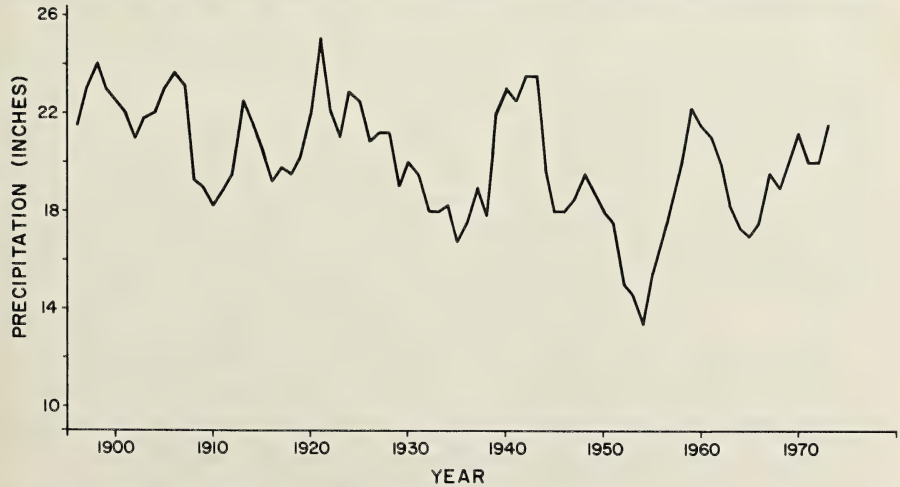


Figure 3. Time series of annual precipitation.

There is an apparent linear trend indicating decreasing average annual precipitation since the turn of the century, but this may be local and not indicative of an overall downward trend on a large scale.

The drought period of the 30's and the even more severe drought of the 50's (for the southern plains) are evident along with periods of above average precipitation in the early 40's, early 20's and around the turn of the century. Another maximum, which occurred in the late 50's and early 60's signalled recovery from the drought, but was not as prominent with regard to total precipitation as those of earlier years.

SPATIAL DISTRIBUTION OF PRECIPITATION

Fig. 4 shows the distribution of mean annual precipitation in the Texas Plains for the 30-yr period from 1944 to 1973. The analysis is based on 52 precipitation reporting stations which are shown in the figure. It is apparent that rainfall decreases from east to west across the area, although not at a uniform rate.

It is important to realize that the sequence of meteorological events leading to precipitation in one season of the year are not the same as those producing precipitation at other times of the year (Haragan, 1976). Precipitation during April and May is usually due to violent convective activity set off by frontal or upper air disturbances. Once the vertical motion is provided, precipitation usually results. Summer rains are generally scattered shower developments which depend mainly on daytime heating, low level moisture and an absence of subsidence aloft. Late autumn and early winter rains may result from warm, moist Gulf air overrunning continental polar air which has been swept southward ahead of a strong winter anticyclone.

Table 1 gives the mean monthly precipitation and standard deviation for 21 representative stations. Fig. 5, which is based on the data in Table 1 plus 31 additional stations, illustrates the spatial distribution of mean monthly precipitation for the rainy period, April through September. While the general east to west decrease remains evident in all months, there are some interesting features in the precipitation patterns worthy of further consideration.

The April map indicates a rather smooth transition across the area and, in fact, shows much the same pattern as the annual distribution shown in Fig. 4. In May, however, the gradients tighten considerably and the tongue of maximum precipitation in the south central region across Plainview (PVW) sharpens and becomes a significant feature. The dominant precipitation maximum east of Miami (MIA) is probably related to topography and should be explored in more detail.

In June, a precipitation maximum appears just southeast of Amarillo. The location of this feature, downwind of an area of heavy irrigation, suggests the low-level moisture increase related to irrigation as a cause. This possibility is particularly interesting in view of the recent results reported by Schickedanz (1976) indicating that irrigation has been an important factor in determining

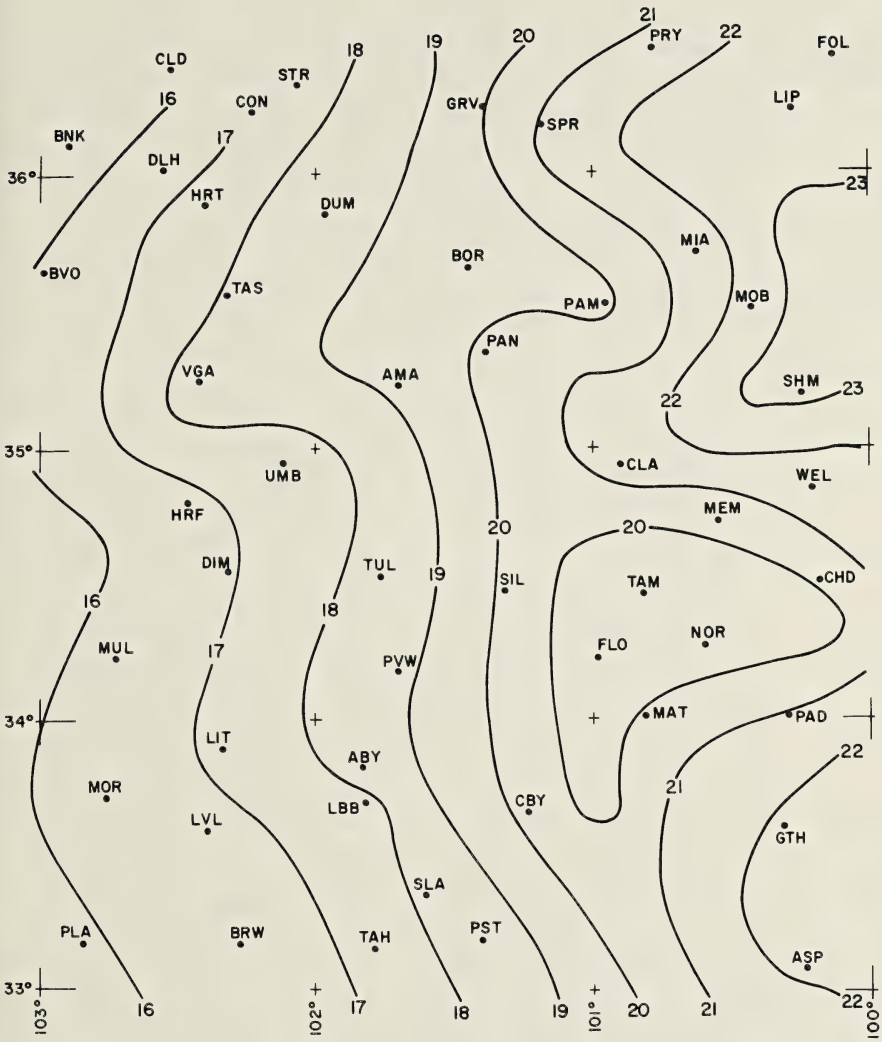


Figure 4. Mean annual precipitation.

precipitation distribution in the Great Plains. Since irrigation has been significant in the Texas High Plains throughout the period of this investigation, it is assumed to be a part of the natural variability. The possibility that an "irrigation bias" is present is currently being investigated.

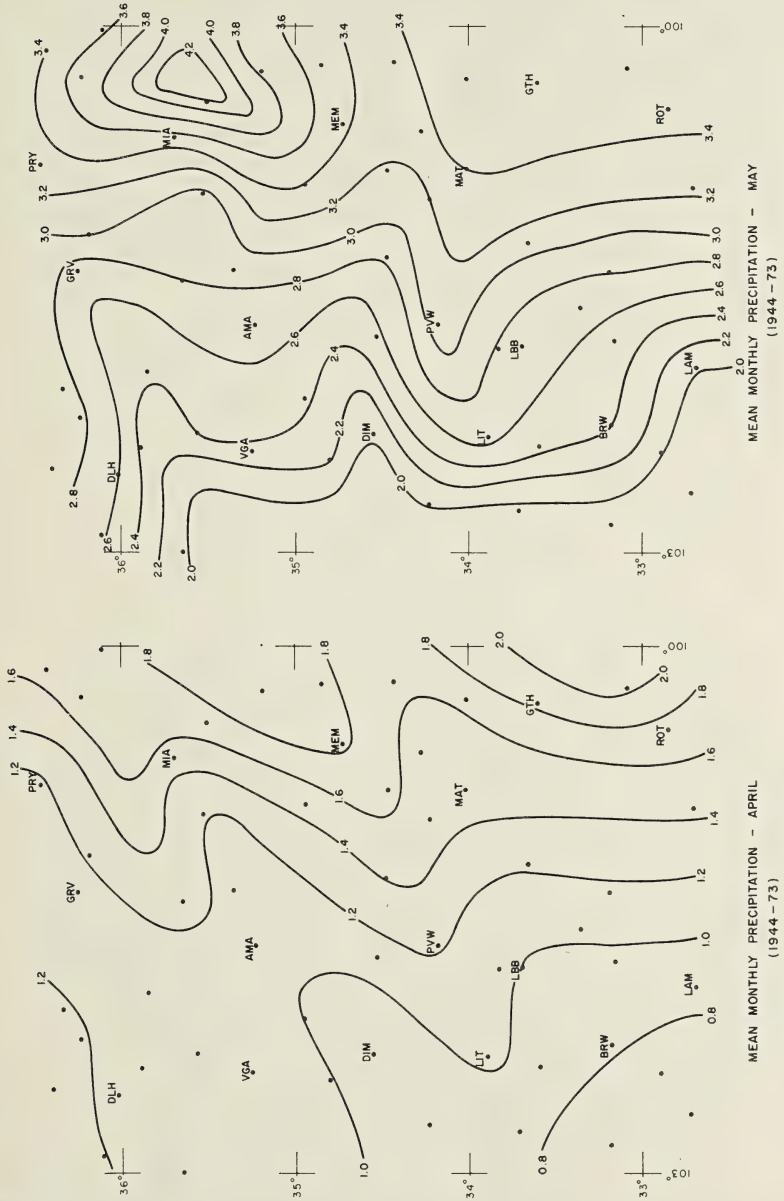
The maps for July and August reflect the scattered nature of the precipitation which characterizes the summer season. The September map reveals minimum rainfall over Lubbock and northwest of Memphis, situations which are probably related to the geometry of tropical air inflow as well as topography.

Further insight into the nature of the spatial distribution is offered by space autocorrelation analysis. Correlation coefficients utilizing more than 1300 station-pairs have revealed the seasonal nature of the precipitation in the Texas High

TABLE 1

Mean Monthly Precipitation

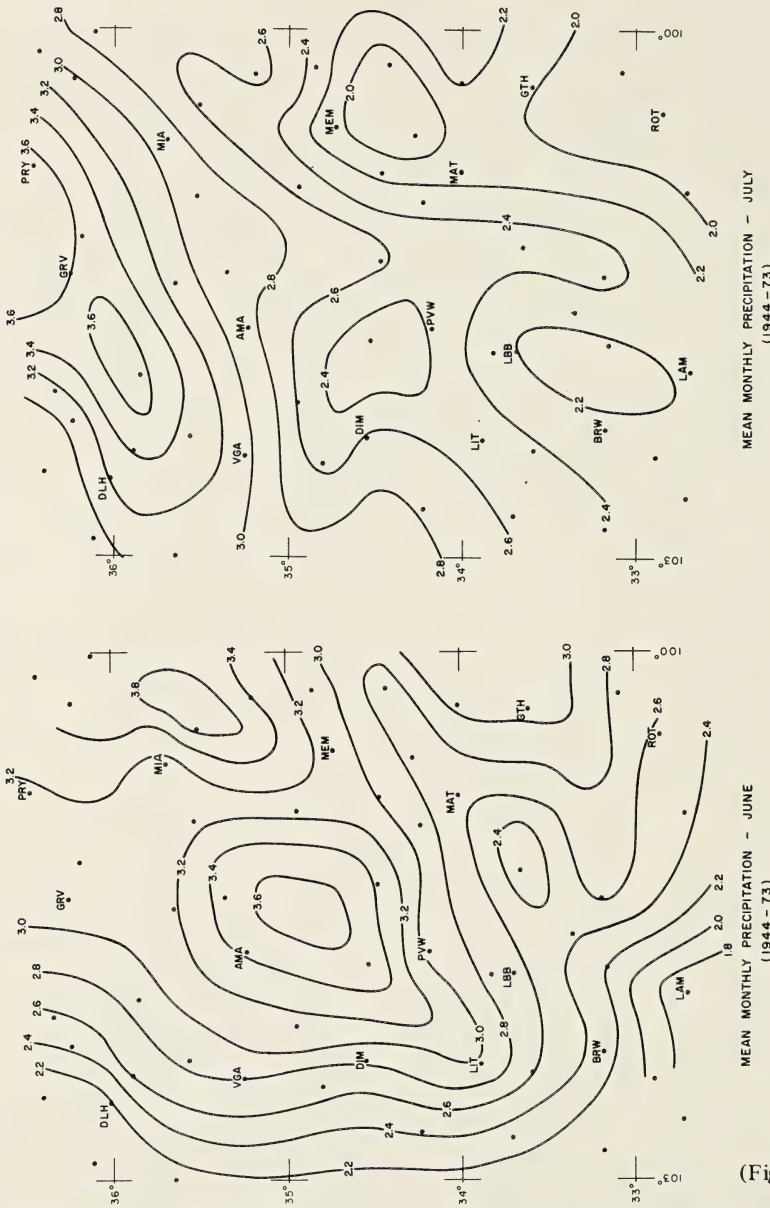
Station		J	F	M	A	M	J	J	A	S	O	N	D
Amarillo	$\bar{P}$	0.55	0.58	0.81	1.12	2.66	3.47	2.87	2.87	1.93	1.52	0.62	0.58
	σ	0.60	0.48	1.00	0.79	2.04	2.63	2.08	1.45	1.44	1.46	0.68	0.83
Borger		0.50	0.73	0.82	1.34	2.90	3.03	3.04	2.48	1.78	1.33	0.72	0.56
		0.58	0.69	1.12	1.04	1.93	2.03	2.32	1.47	1.33	1.27	0.74	0.70
Bravo		0.40	0.45	0.61	1.09	2.09	2.17	3.13	2.57	1.68	1.00	0.56	0.51
		0.46	0.52	0.77	1.18	1.28	2.17	1.52	1.48	1.44	1.06	0.68	0.76
Childress		0.76	0.74	1.05	1.64	3.31	2.79	1.97	1.87	2.26	2.01	0.75	0.75
		0.91	0.65	1.07	1.46	2.20	1.93	1.71	1.50	1.89	1.74	0.74	0.92
Crosbyton		0.74	0.73	0.97	1.29	3.17	2.31	2.48	2.12	2.56	2.27	0.82	0.73
		0.85	0.61	0.96	1.14	2.41	1.57	1.82	1.80	2.11	2.10	0.92	0.79
Dalhart		0.35	0.52	0.72	1.16	2.62	2.11	3.30	2.47	1.43	1.23	0.55	0.41
		0.36	0.50	0.88	1.07	1.53	1.46	2.03	1.57	1.23	1.49	0.71	0.46
Hereford		0.49	0.55	0.62	1.03	2.22	2.78	2.57	2.29	1.59	1.56	0.57	0.46
		0.58	0.52	0.89	0.72	2.09	1.83	1.99	1.46	1.16	1.47	0.70	0.84
Higgins		0.55	0.99	1.20	1.77	3.53	3.59	2.76	2.47	2.34	1.92	0.92	0.70
		0.69	1.07	1.37	1.23	2.64	2.29	2.10	1.45	1.60	1.59	1.13	0.68
Lamesa		0.74	0.37	0.72	0.83	2.05	1.70	2.26	1.46	2.34	1.80	0.61	0.45
		0.83	0.44	0.92	0.80	1.75	1.13	1.95	1.49	1.85	1.35	0.71	0.63
Lubbock		0.58	0.55	0.82	0.99	2.75	2.71	2.20	1.95	1.98	1.91	0.47	0.47
		0.80	0.62	0.89	0.87	1.94	2.09	1.49	1.84	1.74	1.89	0.64	0.47
Matador		0.65	0.64	0.89	1.50	3.02	2.94	2.31	2.06	2.35	2.25	0.79	0.76
		0.82	0.56	1.14	1.50	2.22	2.22	2.02	1.46	2.05	2.07	0.82	0.93
Memphis		0.75	0.81	0.92	1.80	3.57	3.08	2.07	1.92	2.34	1.83	0.67	0.64
		0.92	0.67	1.12	1.59	2.41	2.98	1.41	1.75	1.79	1.75	0.76	0.91
Miami		0.55	0.80	0.95	1.56	3.46	3.01	2.93	2.61	2.18	1.72	0.90	0.70
		0.62	0.69	1.17	1.44	2.37	1.83	1.95	1.37	1.80	1.49	1.19	1.03
Morton		0.46	0.39	0.49	0.86	1.86	2.27	2.59	2.40	2.54	1.64	0.45	0.35
		0.56	0.36	0.76	0.91	1.40	1.62	2.40	2.21	2.14	1.61	0.55	0.39
Muleshoe		0.58	0.49	0.49	0.86	2.10	2.51	2.77	2.17	1.99	1.40	0.55	0.41
		0.60	0.40	0.65	0.76	1.81	1.74	2.01	1.70	1.42	1.49	0.65	0.41
Plainview		0.72	0.62	0.75	1.24	3.10	2.94	2.43	1.88	2.13	1.60	0.52	0.63
		0.87	0.57	0.83	1.21	2.37	1.75	2.34	1.30	1.70	1.58	0.53	0.72
Seminole		0.64	0.58	0.71	0.93	1.96	1.53	2.22	2.09	2.12	1.52	0.44	0.36
		0.84	0.72	1.01	1.05	1.90	1.16	1.67	1.94	1.74	1.47	0.58	0.38
Shamrock		0.66	0.90	1.17	1.92	3.75	3.40	2.73	2.56	2.54	1.95	0.81	0.70
		0.78	0.89	1.37	1.71	2.63	2.07	2.33	1.72	1.93	1.72	0.89	0.94
Snyder		0.65	0.63	0.72	1.55	3.38	2.45	1.99	2.16	2.33	2.10	0.87	0.66
		0.77	0.64	0.78	1.34	2.22	1.77	1.84	3.21	1.76	1.47	1.12	0.72
Spearman		0.57	0.75	1.20	1.23	3.08	3.10	3.64	2.62	1.94	1.52	1.02	0.59
		0.58	0.59	1.58	0.96	2.06	1.96	2.27	1.67	1.90	1.79	1.28	0.75
Vega		0.55	0.68	0.82	1.15	2.39	2.82	3.15	2.63	1.74	1.34	0.70	0.60
		0.63	0.62	0.87	0.94	2.02	2.68	2.05	1.27	1.28	1.28	0.88	0.78



(Figure 5)

Plains (Haragan, 1976). An example of this type of analysis is given by Figs. 6 and 7 which show correlation as a function of separation distance. Although significant scatter is evident, the curve for April shows a linear relationship indicative of the organized nature of precipitation during this month of the year; once upward vertical motion is provided, abundant precipitation falls over a wide area

(Figure 5, cont.)



(Figure 5)

along the path of the storm. Considerably greater scatter is shown in August when local showers due to surface heating and convective instability produce high precipitation gradients and smaller correlations among stations.

ANALYSIS OF PRECIPITATION EVENTS

Results of a North Dakota experiment to increase precipitation by cloud-seeding revealed a greater number of rainfall events during seeding periods. More rainfall

(Figure 5, cont.)

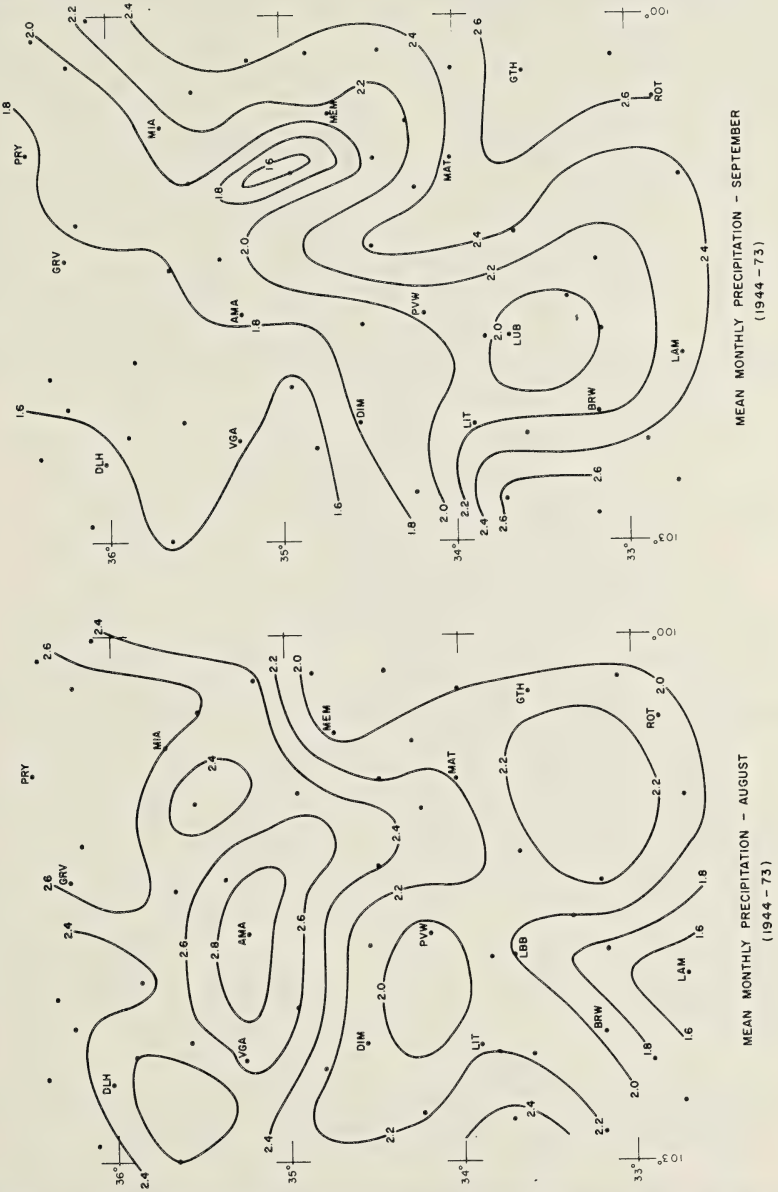


Figure 5. Spatial distribution of mean monthly precipitation for the rainy period, April through September.

and a larger proportion of large rain events were positively correlated with seeding (Schleusener and Miller, 1974).

In order to test this hypothesis for cloud-seeding projects in Texas, it is necessary to establish a climatology of rainfall events in the absence of seeding. Tables 2 and 3 summarize the required data for 7 stations in the High Plains area.

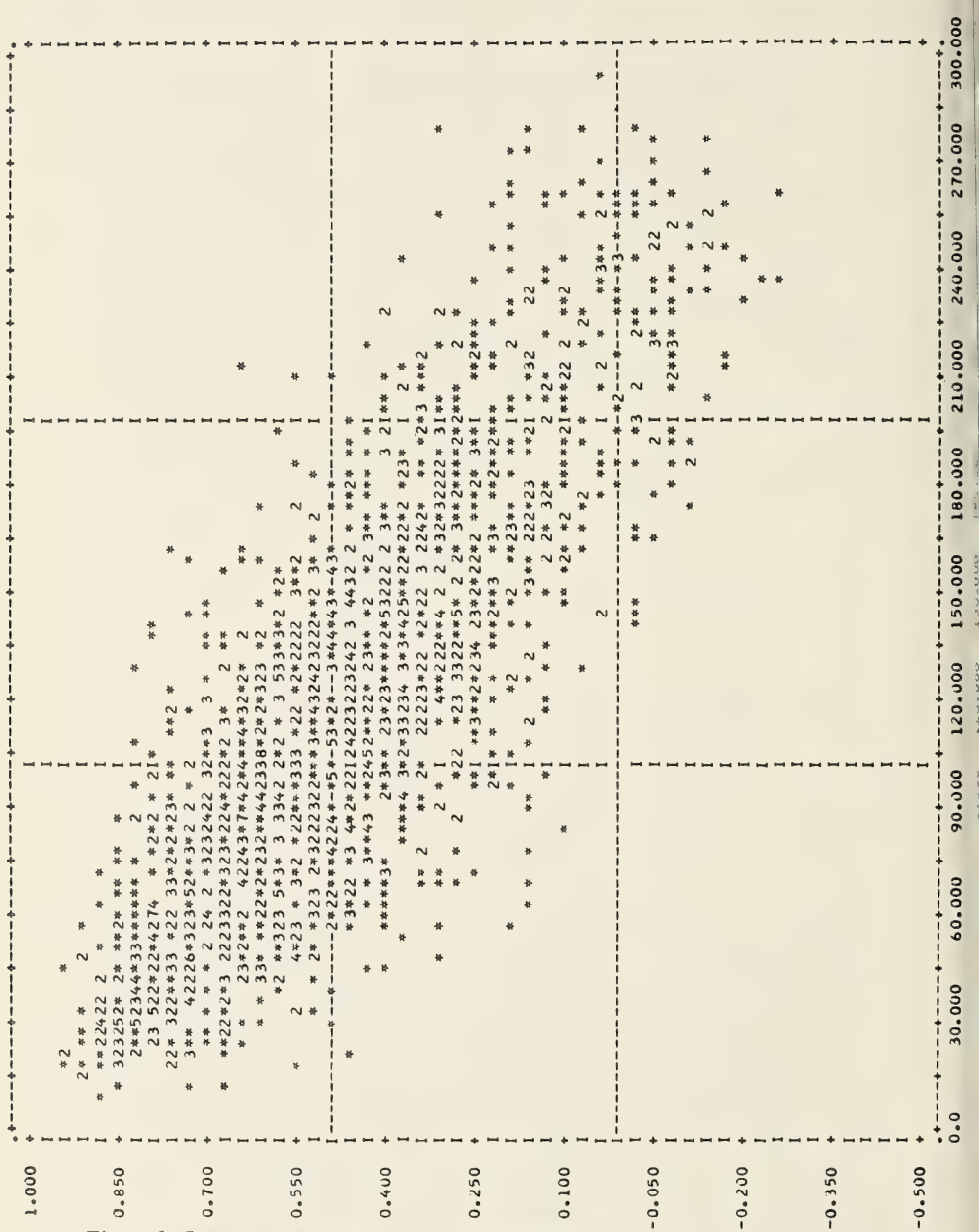


Figure 6. Space autocorrelation – May 1944-1973.

Table 2 shows the % frequency of various numbers of rainfall periods per month based on the total period of record at each station. A rainfall period refers to a sequence of days all having a measurable amount of rain. Thus, the 10-day sequence of rainfall:

1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
0	0	.05	.08	0	0	1.06	0	0	0

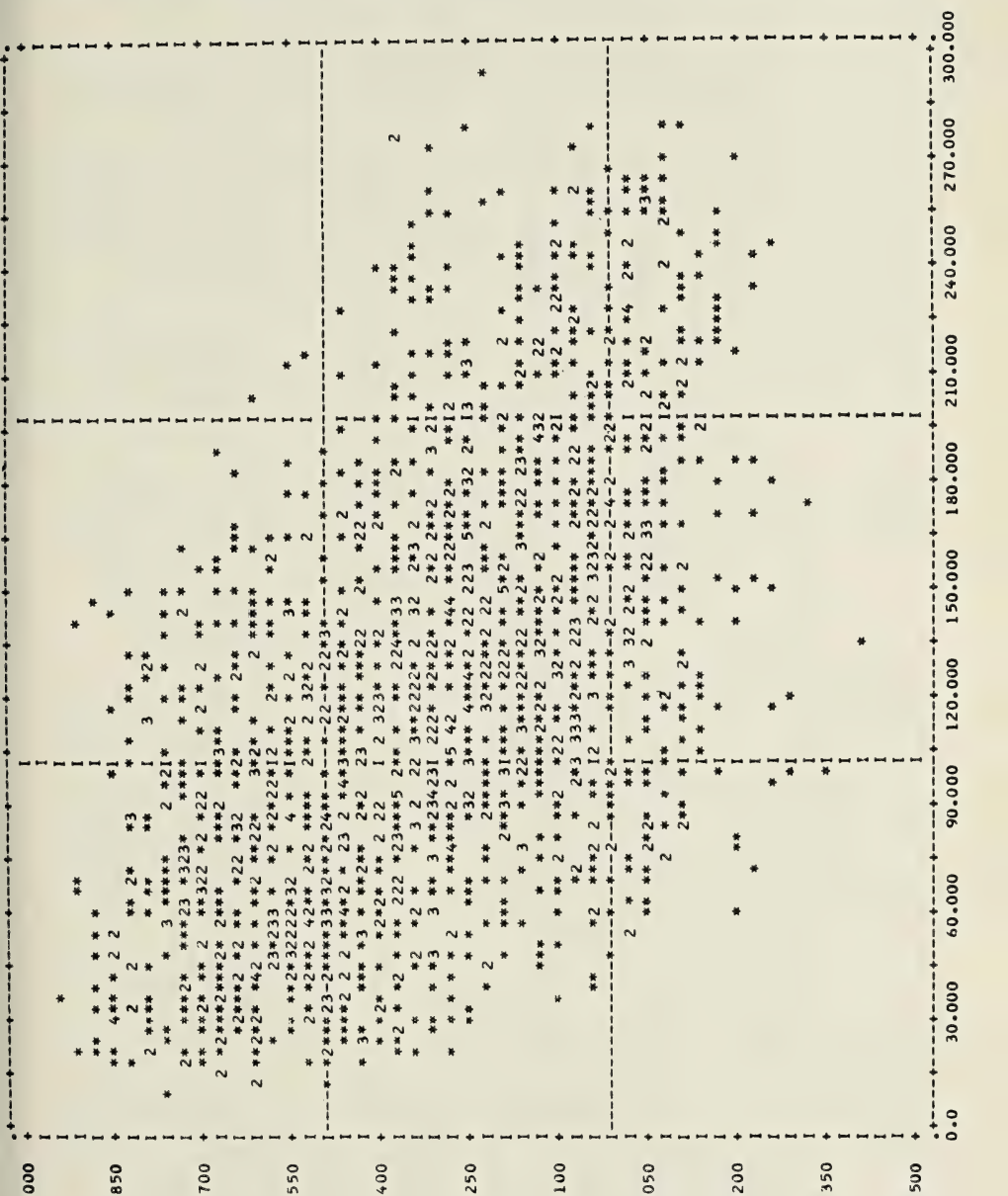


Figure 7. Space autocorrelation – August 1944-1973.

contains 2 rainfall periods. In considering the number of rainfall periods per month, a period extending into the next month counts only for the month in which it began. It is evident from Table 2 that during some individual months there were no rainfall periods, whereas during others, more than 7 periods were observed. As expected, the average number per month was greatest in summer and lowest in winter.

TABLE 2
% Frequency of Rainfall Periods

Station	Rainfall Periods Per Month	J	F	M	A	M	J	J	A	S	O	N	D
Amarillo	0	9	4	6	3	0	0	0	0	0	3	11	9
(1892)	1	14	23	17	9	1	3	0	0	4	10	24	11
	2	24	24	15	22	6	10	9	6	19	24	25	28
	3	26	21	26	24	7	13	9	17	26	32	25	25
	4	16	17	19	19	23	19	35	26	26	19	8	18
	5	10	8	8	8	32	29	19	26	13	10	3	9
	6	1	2	5	10	21	18	21	18	9	1	1	0
	7	0	0	3	4	10	7	5	6	2	0	3	0
	>7	0	1	1	1	0	1	2	1	1	1	0	0
Crosbyton	0	8	9	9	0	0	0	0	2	0	2	14	12
(1892)	1	27	25	21	17	2	9	7	7	7	16	23	24
	2	24	26	16	29	14	16	14	12	24	14	30	16
	3	15	19	26	29	12	10	23	19	27	28	18	33
	4	12	18	19	9	12	33	29	17	26	23	9	10
	5	14	3	5	12	29	19	15	29	14	16	5	3
	6	0	0	4	2	24	7	7	11	2	1	1	2
	7	0	0	0	2	7	6	3	3	0	0	0	0
	>7	0	0	0	0	0	0	2	0	0	0	0	0
Dalhart	0	24	9	12	1	3	0	0	0	1	6	22	12
(1906)	1	30	20	14	11	2	0	1	1	21	17	28	25
	2	19	31	23	21	10	13	6	7	12	27	25	25
	3	21	21	21	30	15	10	16	21	18	30	20	27
	4	4	14	14	29	24	24	22	18	26	11	2	8
	5	2	5	13	3	25	26	21	25	8	8	2	3
	6	0	0	3	2	15	18	21	16	12	1	1	0
	7	0	0	0	3	5	7	12	12	2	0	0	0
	>7	0	0	0	0	1	2	1	0	0	0	0	0
Lamesa	0	15	16	9	9	2	2	0	3	1	6	25	22
(1910)	1	23	26	38	22	14	10	13	14	19	21	26	30
	2	27	23	21	33	13	25	21	34	25	25	24	22
	3	19	22	24	20	22	28	27	14	23	21	14	10
	4	13	9	5	5	22	14	24	13	14	22	9	13
	5	3	2	3	8	11	16	13	9	11	3	0	3
	6	0	2	0	2	13	5	1	8	5	2	2	0
	7	0	0	0	1	3	0	1	2	2	0	0	0
	>7	0	0	0	0	0	0	0	3	0	0	0	0
Lubbock	0	8	5	13	0	2	2	3	2	2	3	13	8
(1912)	1	30	22	21	20	2	2	9	6	4	17	23	19
	2	25	30	22	31	9	12	9	9	19	16	25	25
	3	18	25	19	24	14	27	13	14	30	31	23	20
	4	14	11	17	14	31	20	32	26	23	17	8	20
	5	5	3	8	9	23	22	17	19	19	13	6	6
	6	0	2	0	0	11	9	11	22	3	3	2	0
	7	0	2	0	2	3	6	3	2	0	0	0	2
	>7	0	0	0	0	5	0	3	0	0	0	0	0
Miami	0	14	12	8	0	0	1	2	0	3	3	15	10
(1906)	1	28	24	14	9	0	5	5	3	6	18	25	21
	2	21	20	25	20	9	5	8	14	23	24	26	31
	3	23	27	21	31	21	25	26	20	26	28	26	26
	4	9	11	14	17	24	20	23	23	26	13	5	12
	5	5	4	11	12	20	23	20	23	10	6	2	0
	6	0	2	5	8	20	12	12	8	6	6	0	0
	7	0	0	2	3	3	8	4	8	0	2	1	0
	>7	0	0	0	0	3	1	0	1	0	0	0	0
Plainview	0	11	12	9	3	0	0	0	0	4	4	17	11
(1894)	1	32	22	27	17	2	7	5	5	11	20	26	29
	2	23	31	23	34	9	14	12	8	17	24	30	17
	3	15	17	19	20	22	15	26	21	26	26	15	21
	4	14	14	14	17	20	31	23	17	17	12	6	15
	5	5	3	6	5	25	11	19	26	21	5	4	6
	6	0	0	2	3	12	15	12	9	1	9	2	1
	7	0	0	0	1	9	5	3	9	3	0	0	0
	>7	0	1	0	0	1	2	0	5	0	0	0	0

TABLE 3
Summary of Rainfall Amounts Per Period

Station	Inches Per Rainfall Period	J	F	M	A	M	J	J	A	S	O	N	D
Amarillo	0.00-0.24	154	147	158	152	181	156	168	149	140	117	100	139
(1892)	0.25-0.49	25	27	35	37	37	53	51	69	49	35	26	34
	0.50-0.74	13	15	20	23	49	42	50	46	31	28	20	15
	0.75-0.99	6	7	7	13	34	24	29	14	13	14	6	8
	1.00-1.49	1	7	6	26	36	34	20	23	21	21	13	5
	1.50-1.99	3	2	3	8	12	18	21	26	15	6	3	0
	2.00-2.49	1	1	2	2	11	7	5	14	4	3	2	0
	2.50-2.99	0	0	1	2	5	5	4	4	5	5	1	1
	3.00-3.49	0	0	1	2	3	1	6	3	3	2	2	1
	3.50-3.99	0	0	0	0	1	5	2	3	4	2	0	0
	> 3.99	0	0	0	0	6	5	3	5	2	2	0	1
	TOTAL	203	206	233	265	375	350	359	356	287	235	173	204
Crosbyton	0.00-0.24	83	76	83	81	97	98	91	93	73	73	66	76
(1892)	0.25-0.49	30	25	30	24	54	31	34	44	27	26	21	25
	0.50-0.74	12	12	11	14	37	28	25	26	22	13	11	9
	0.75-0.99	5	8	10	14	22	17	22	13	16	15	6	5
	1.00-1.49	7	4	5	15	20	26	25	24	12	19	11	8
	1.50-1.99	0	2	2	8	11	10	7	11	14	13	1	2
	2.00-2.49	1	0	4	3	13	7	6	2	8	4	2	2
	2.50-2.99	1	0	1	0	3	2	1	8	3	2	0	2
	3.00-3.49	0	0	0	3	0	2	4	2	3	0	0	0
	3.50-3.99	0	0	0	0	2	2	1	1	3	4	0	0
	> 3.99	0	0	0	1	4	1	2	2	5	7	0	0
	TOTAL	139	127	146	163	263	224	218	226	186	176	118	129
Dalhart	0.00-0.24	77	110	115	105	139	154	151	140	115	84	60	88
(1906)	0.25-0.49	16	26	29	36	43	56	60	51	37	29	21	26
	0.50-0.74	8	5	14	17	21	24	35	41	20	18	9	7
	0.75-0.99	2	2	6	17	19	19	18	19	11	6	5	7
	1.00-1.49	0	3	5	15	21	22	24	29	17	12	6	4
	1.50-1.99	2	0	2	8	18	16	11	12	7	6	1	1
	2.00-2.49	0	0	2	0	10	6	7	9	3	1	0	0
	2.50-2.99	0	0	0	0	7	4	7	4	1	2	0	2
	3.00-3.49	0	0	1	1	1	2	2	1	1	2	2	0
	3.50-3.99	0	0	0	0	2	3	2	1	0	3	0	0
	> 3.99	0	0	0	2	4	2	3	1	2	3	0	0
	TOTAL	105	146	174	201	285	308	320	308	214	166	104	135
Lamesa	0.00-0.24	76	76	56	63	98	78	78	78	56	57	48	56
(1910)	0.25-0.49	31	23	24	29	39	45	40	40	44	25	24	22
	0.50-0.74	7	11	18	17	25	17	24	28	24	20	15	14
	0.75-0.99	7	8	9	10	18	17	15	14	14	9	8	4
	1.00-1.49	2	4	5	17	20	17	20	16	17	20	4	8
	1.50-1.99	1	1	2	4	9	10	7	6	8	5	3	1
	2.00-2.49	2	0	3	3	4	5	7	4	8	8	2	2
	2.50-2.99	0	0	0	1	1	2	1	4	6	4	0	0
	3.00-3.49	0	0	0	1	1	1	1	4	1	3	0	0
	3.50-3.99	0	0	0	0	2	0	3	0	2	2	0	0
	> 3.99	0	1	0	0	3	4	2	1	5	3	0	0
	TOTAL	126	124	117	145	220	196	198	195	185	156	104	107

TABLE 3 (Continued)

Station	Inches Per Rainfall Period	J	F	M	A	M	J	J	A	S	O	N	D
Lubbock	0.00-0.24	94	107	78	85	130	100	112	141	95	86	91	109
(1912)	0.25-0.49	24	20	31	36	48	50	51	40	33	29	18	29
	0.50-0.74	8	12	16	12	22	28	24	22	22	23	18	11
	0.75-0.99	6	7	3	13	23	25	19	21	18	12	3	5
	1.00-1.49	1	4	11	12	17	28	26	17	20	15	5	6
	1.50-1.99	0	0	6	9	15	9	7	11	7	4	2	2
	2.00-2.49	1	1	1	2	10	5	3	4	5	2	1	0
	2.59-2.99	1	0	0	1	4	1	4	2	4	7	0	0
	3.00-3.49	0	0	0	1	3	4	0	0	3	2	0	0
	3.50-3.99	0	0	0	0	0	2	0	3	3	0	0	0
	> 3.99	0	0	0	0	4	2	3	1	6	7	0	0
	TOTAL	135	151	146	171	276	254	249	262	216	187	138	162
Miami	0.00-0.24	86	82	89	98	118	95	89	97	90	78	69	82
(1906)	0.25-0.49	20	31	42	37	49	54	46	52	29	32	22	31
	0.50-0.74	13	19	23	26	33	36	38	31	20	24	11	11
	0.75-0.99	4	4	9	18	21	25	25	25	14	14	4	7
	1.00-1.49	4	5	7	25	24	29	29	29	23	14	10	4
	1.50-1.99	3	1	4	9	19	13	14	11	16	9	8	4
	2.00-2.49	0	2	2	5	12	5	9	11	11	6	1	0
	2.50-2.99	0	0	0	0	5	7	2	5	3	5	2	2
	3.00-3.49	0	0	0	2	2	6	3	3	4	3	2	0
	3.50-3.99	0	0	0	0	2	3	1	2	1	0	1	0
	> 3.99	0	1	0	0	6	3	2	2	1	3	1	1
	TOTAL	130	145	176	220	291	276	258	268	212	188	131	142
Plainview	0.00-0.24	85	88	76	80	126	98	111	122	76	67	69	87
	0.25-0.49	27	21	29	28	51	40	39	59	51	36	23	35
	0.50-0.74	4	18	20	15	33	34	28	37	22	21	12	12
	0.75-0.99	7	7	4	16	16	19	22	21	15	20	9	3
	1.00-1.49	6	3	11	14	19	28	24	30	17	12	7	9
	1.50-1.99	2	2	3	10	15	18	10	13	17	8	1	1
	2.00-2.49	1	1	1	4	6	8	7	1	5	5	2	0
	2.50-2.99	0	0	0	4	6	6	6	4	4	6	0	2
	3.00-3.49	0	0	1	0	2	3	1	2	2	1	1	0
	3.50-3.99	0	0	0	1	6	0	2	0	1	1	0	0
	> 3.99	0	1	0	0	5	3	2	1	4	2	0	0
	TOTAL	132	141	145	172	285	257	252	290	214	179	124	149

The rainfall periods tabulated in Table 2 brought widely differing amounts of rain, from a trace to more than 9 in. The size distribution of rains and the frequency of rainfall periods change from month to month. The number of rainfall periods bringing various amounts of rain are shown by month in Table 3.

Most of the rainfall periods brought less than 1/2 in of rain. Larger rains are much less common. Nearly all of the big rains (2 in or more) occur in the warm season, May to October. These big rains are distinctive features of rainfall on the High Plains and have a considerable influence on the average precipitation (Covey, *et al.*, 1957). In spite of their importance, they are infrequent happenings. Approximately 100 occurred in Lubbock over a 61-yr period while about 150 occurred at Amarillo in 81 years.

APPLICATION TO WEATHER MODIFICATION VALUATION

Weather modification technology has developed to the extent that it is being applied by a significant number of groups in Texas and throughout the Great Plains. However, the state of the art is such that all efforts whether scientific or operational should be considered experimental (Haragan, 1974). All projects are carried out in the face of uncertainties which still remain on all scales of weather systems, particularly for larger areas (Schleusener, *et al.*, 1974).

One of the key problems encountered in precipitation modification experiments has been the evaluation of results. Evaluation of modest increases or decreases in surface precipitation resulting from cloud-seeding operations is exceedingly difficult because of the great natural variability of precipitation in space and time. Several evaluation techniques are based upon a knowledge of the precipitation climatology of the region in question. One method involves computing the % differences between seeded and historical periods. In order to further assess seeding effectiveness, ratios of target average rainfall to surrounding "control areas" can be computed for the historical and seeded periods.

Another approach involves the correlation between the rainfall in target and control areas. Changing correlation patterns may be related to the presence of an outside influence on the distribution and amount of precipitation. Depending upon the magnitude of the correlations, prediction equations can be derived and used to estimate the amount of rainfall expected in the target area during seeding periods. Cloud-seeding effectiveness can thus be measured by computing the difference between the actual and expected (estimated from the regression equation) precipitation. Further insight can be derived by considering the distribution of rainfall events and the amount of precipitation associated with these events during seeded and historical periods both inside and outside of the target area.

Huff (1971) has pointed out that the problem of weather modification evaluation has been compounded by the lack of adequate statistical data to define quantitatively the natural variability characteristics in most regions of the United States. This investigation provides the climatological data necessary to evaluate long-term weather modification experiments in the Texas High Plains.

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STRUCTURAL GEOLOGY OF THE EDWARDS AQUIFER IN THE CASTLE HILLS QUADRANGLE, NORTHWESTERN SAN ANTONIO, TEXAS

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ABSTRACT

The Edwards Aquifer is comprised of the Edwards Group and the Walnut and Georgetown Formations in the northwestern San Antonio area. In this area the aquifer exists in a series of discrete fault blocks. The aquifer is more or less continuous from block to block. The edges of the blocks are, in a few instances, apparently bent, but the apparent warping probably results from a large number of small displacement faults successively offsetting the aquifer units. These areas may be favorable sites for obtaining large well yields.

INTRODUCTION

The Castle Hills Quadrangle includes about 65 sq mi of northwestern San Antonio, Texas, and several suburbs. The area is cut by faults of the Balcones fault zone, a set of synthetic, normal faults most of which are displaced down toward the Gulf of Mexico Basin. The Castle Hills Quadrangle is underlain by Lower and Upper Cretaceous carbonate rock units and a few, relatively much thinner interbedded units of cretaceous clay or shale (Fig. 1). The Georgetown Formation, Edwards Group, and Walnut Formation comprise the Edwards Aquifer[1] in this part of the Balcones fault zone. The surface distribution of the rocks comprising this aquifer and their surface and subsurface structural relations are presented here. This study includes additions to and refinements in the detailed geology and the delineation of the recharge area previously mapped by Reeves (1971).

AREAL GEOLOGY

The rocks of the Castle Hills Quadrangle are cut by numerous faults that are usually downthrown to the southeast so that successively younger rocks crop

[1]This sequence is equivalent in this area to the Edwards and associated limestones formerly mapped by the U.S. Geological Survey.

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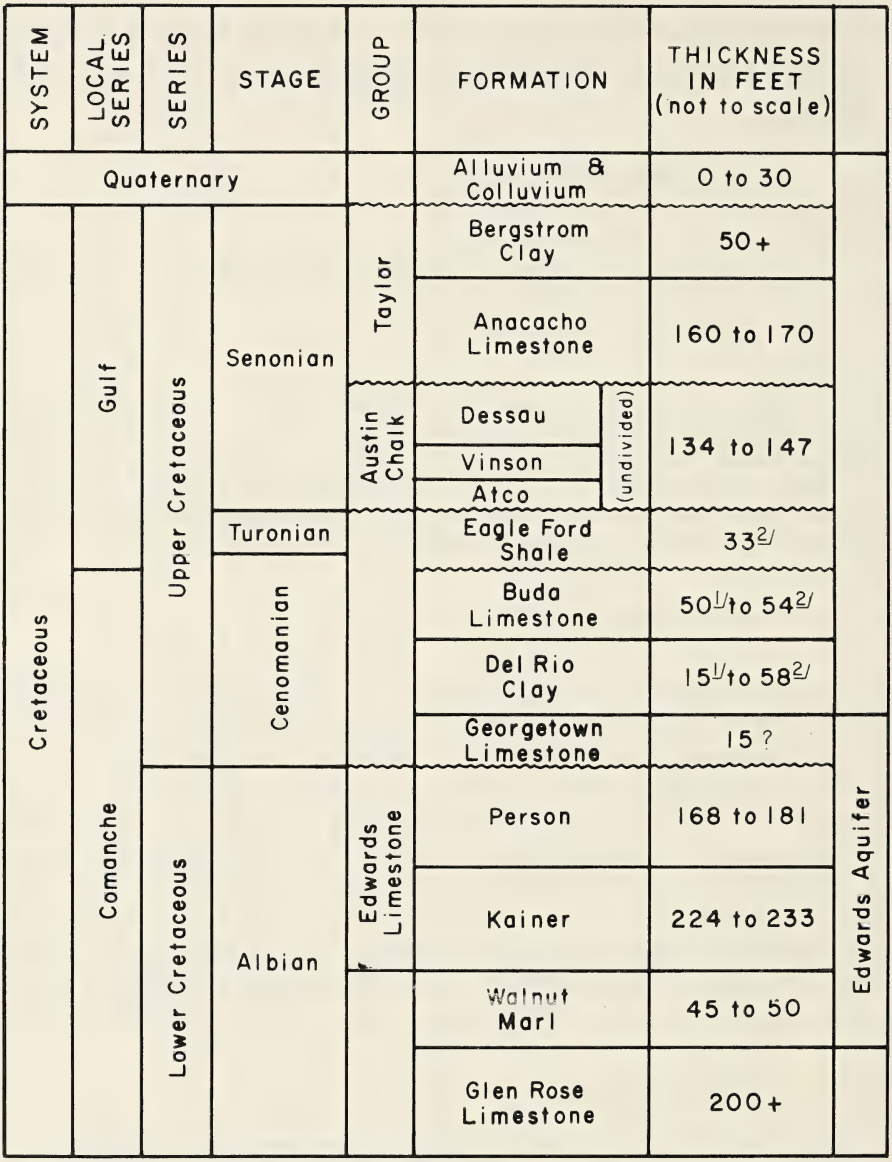


~~~~ Disconformity.

1/ Minimum thickness of a "complete" exposure. Thinning has occurred by surficial slumping and/or by downhill creep.

2/ The undisturbed thickness as shown in well logs in the area.

Figure 1. Stratigraphic column for the Castle Hills Quadrangle.
~~~~

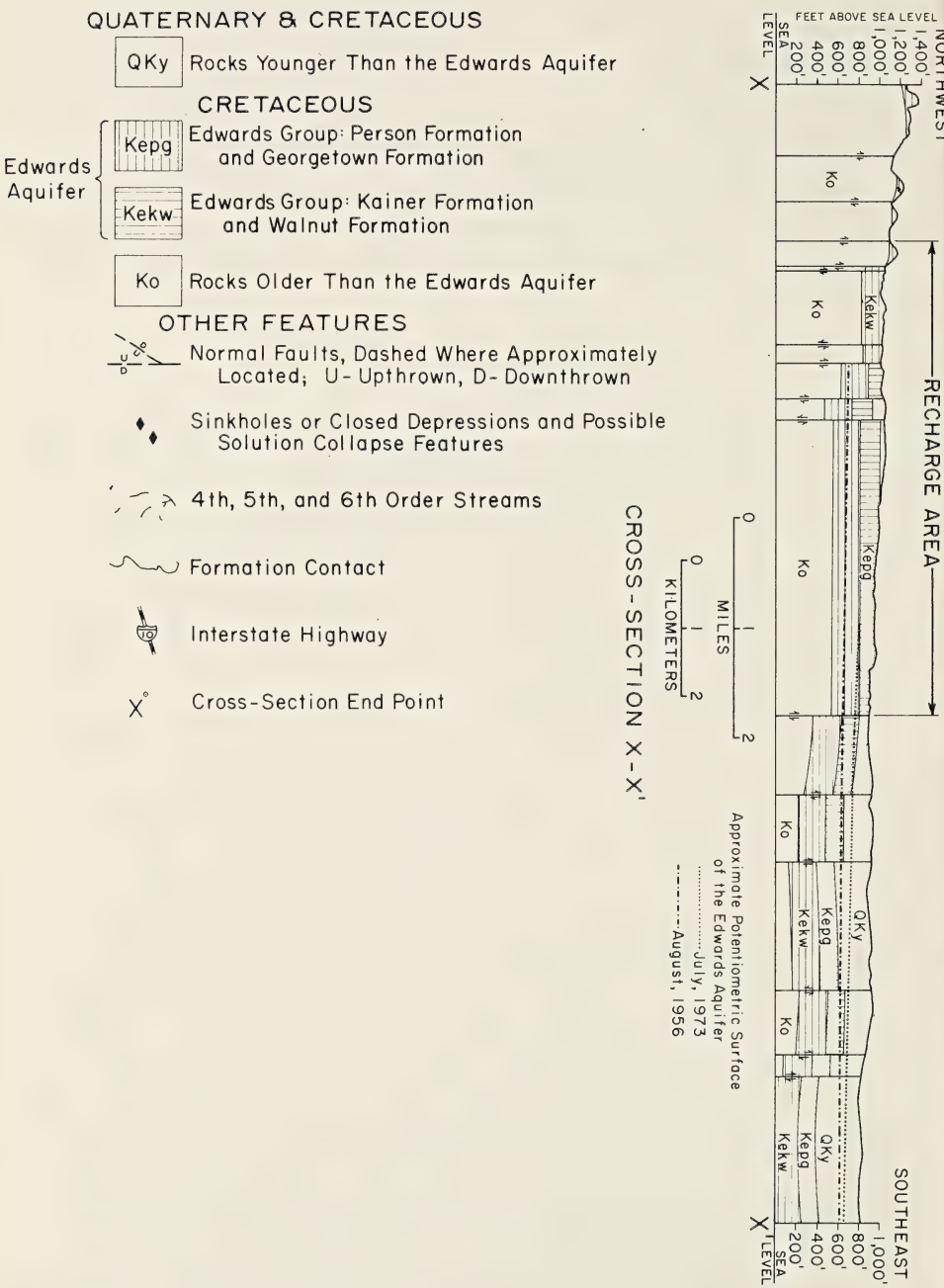
out from northwest to southeast. Rocks of the Edwards Aquifer crop out in a broad band across the northern 1/3 of the quadrangle, with older rocks occurring in the extreme northwestern corner of the area and younger rocks covering the remainder of the quadrangle. The outcrop area of the units comprising the Edwards Aquifer is part of the recharge area for the aquifer (Fig. 2). The Georgetown and Walnut Formations are thin relative to the Edwards Group and are not shown separately on the map of the recharge area. The Kainer Formation (lower Edwards Group and Walnut Formation are mapped as a unit (Kekw) on Fig. 2. Similarly, the Person Formation (upper Edwards Group) and Georgetown Formation are mapped as a unit (Kepg).

STRUCTURAL GEOLOGY

Structural contours showing the elevation of the top of the Edwards Aquifer (top of Georgetown Formation) were drawn on data from drillers' logs and scattered geophysical logs (Fig. 3). The term "Edwards" is often the only description recorded by water well drillers for the hard limestones containing red clay, chert, and numerous cavities, which characteriz. most of the rocks of the Edwards Aquifer. The top of the "Edwards" is often picked by drillers. This is usually at the base of the distinctive Del Rio Clay (top of the Georgetown Limestone) and is easily recognizable because it occurs below a clay–limestone–clay (Eagle Ford–Buda–Del Rio) sequence.

The Buda Formation or the Austin Group may be identified as "Edwards" by drillers if these units are water-bearing. For this reason drillers' logs must be checked closely considering first the well location, surface geology, and nearby well logs and then the occurrence, lithology, and thickness of the Eagle Ford–Buda–Del Rio sequence. A reasonable log would have descriptions of near surface material that agree with observed surface outcrops, and the top of the "Edwards" would occur below a sequence of 30 to 35 feet of clay, shale, or "lignite" (Eagle Ford), 50 to 55 ft of limestone (Buda), and 50 to 60 ft of yellow or blue clay or shale (Del Rio) or an appropriate part of this sequence, depending on surface outcrops.

The outcropping rocks of the Edwards Aquifer units appear to be basically flat-lying though they are broken by numerous faults. The rocks dip generally less than 1° in various directions (Shaw, 1974). In addition, little deformation is discernible from available well data within individual fault blocks (Fig. 3). The contours of the top of the Edwards Aquifer appear to indicate that the aquifer is warped or bent in a few areas adjacent to major faults. There are at least 3 possible ways this apparent warping or bending may be accomplished. The Edwards may be (1) folded downward, (2) comprised of one or more dipping blocks between faults, or (3) successively offset by many small displacement faults. The last of these is most probable here. Surface mapping on the Edwards outcrop showed that the Edwards is a hard, intensely fractured limestone with little or no evidence of folding. The Edwards is such a brittle and competent limestone sequence that


QUATERNARY & CRETACEOUS
QKy
Rocks Younger Than the Edwards Aquifer
CRETACEOUS
Edwards Aquifer
Kepg
Edwards Group: Person Formation and Georgetown Formation
Kekw
Edwards Group: Kainer Formation and Walnut Formation
Ko
Rocks Older Than the Edwards Aquifer
OTHER FEATURES
U
D
Normal Faults, Dashed Where Approximately Located; U- Upthrown, D- Downthrown
Sinkholes or Closed Depressions and Possible Solution Collapse Features
4th, 5th, and 6th Order Streams
Formation Contact
Interstate Highway
Cross-Section End Point
CROSS-SECTION X-X'
NORTHWEST
SOUTHEAST
FEET ABOVE SEA LEVEL
SEA LEVEL
200'
400'
600'
800'
1,000'
1,200'
1,400'
RECHARGE AREA
Ko
Kekw
Kepg
QKy
MILES
KILOMETERS
Approximate Potentiometric Surface of the Edwards Aquifer
July, 1973
August, 1956

(Figure 2)

(Figure 2, cont.)

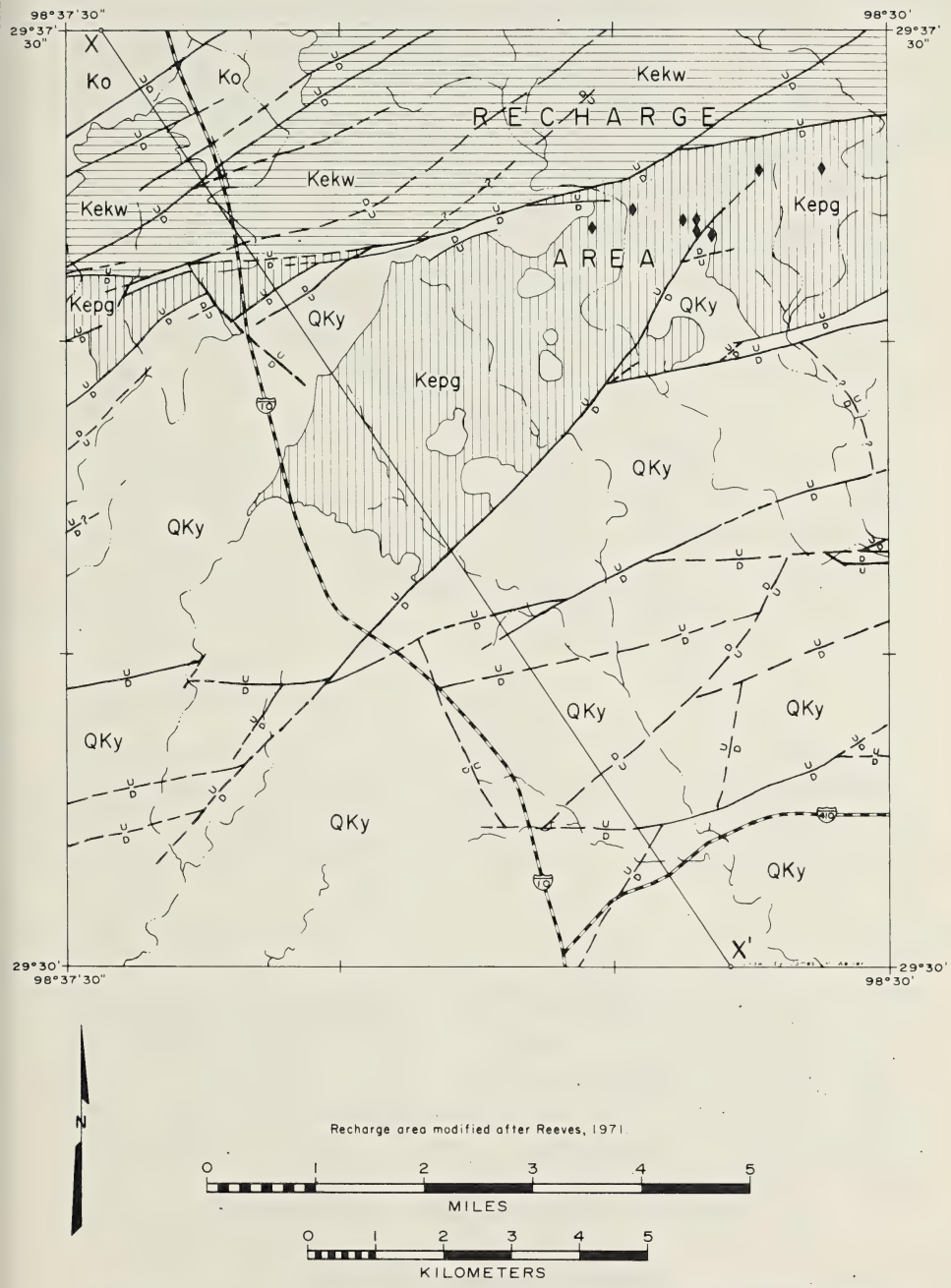


Figure 2. Generalized geologic map and cross section showing recharge area for Edwards Aquifer, Castle Hills Quadrangle.

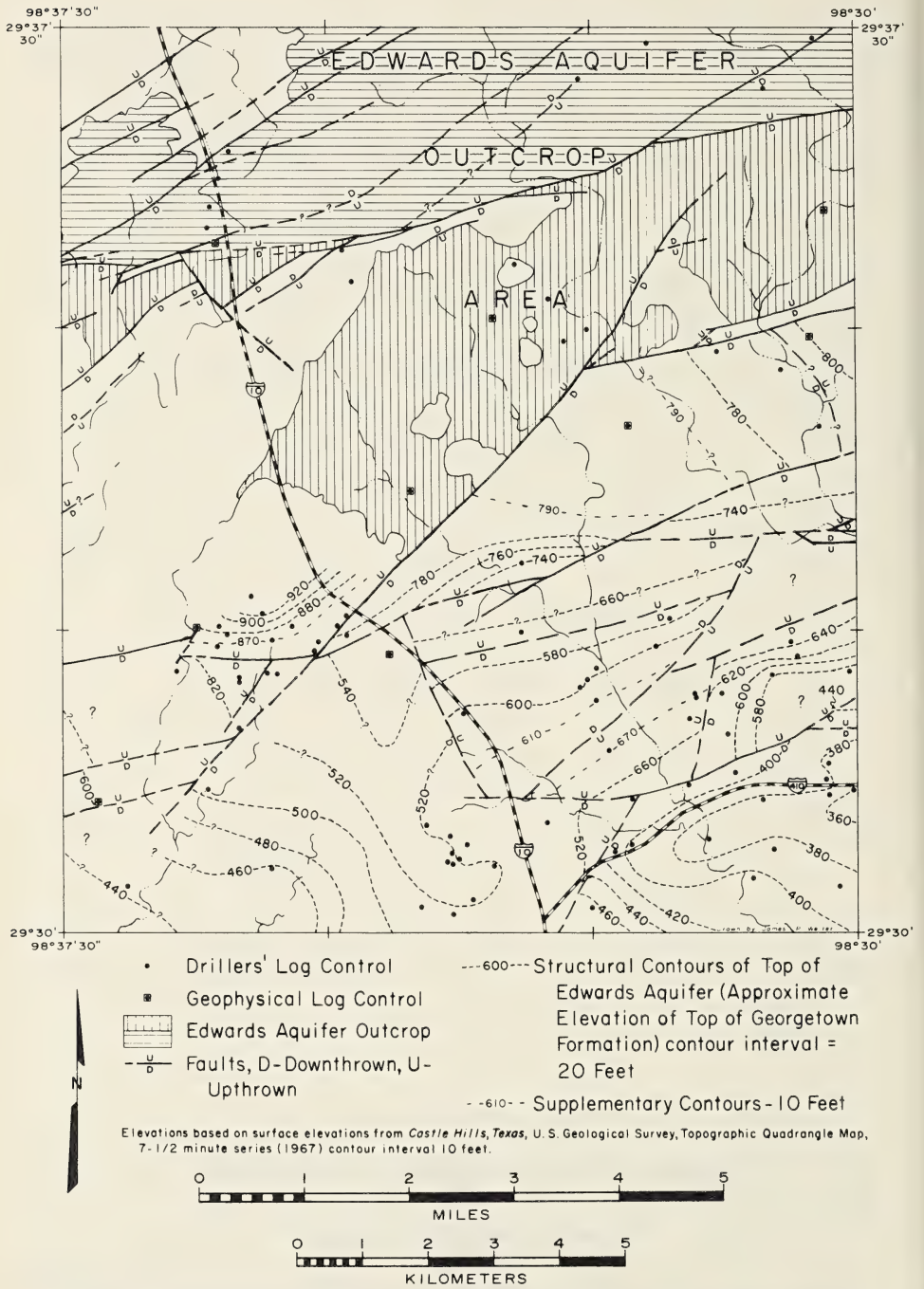

98°37'30"
29°37'30"
98°30'
29°30'
EDWARDS AQUIFER
OUTCROP
AREA
Drillers' Log Control
Geophysical Log Control
Edwards Aquifer Outcrop
Faults, D-Downthrown, U-Upthrown
---600--- Structural Contours of Top of Edwards Aquifer (Approximate Elevation of Top of Georgetown Formation) contour interval = 20 Feet
- -610- - Supplementary Contours - 10 Feet
N
Elevations based on surface elevations from Castle Hills, Texas, U. S. Geological Survey, Topographic Quadrangle Map, 7-1/2 minute series (1967) contour interval 10 feet.
0 1 2 3 4 5
MILES
0 1 2 3 4 5
KILOMETERS

Figure 3. Structural contours of the top of the Edwards Aquifer, Castle Hills Quadrangle.

it is very likely that a large number of small displacement faults successively displace the Edwards wherever it appears to be warped. Surface exposures and cores of the Edwards are usually intensely fractured, showing attendant evidence of solution and precipitation by circulating ground water (Shaw, 1974). In areas of the subsurface, where rocks of the Edwards Aquifer appear to be warped, they are probably extremely fractured and modified by solution activity. Such areas are potential locations for high yield wells if enough saturated thickness of aquifer is available.

Well control was also used to substantiate major fault displacements where the Edwards Aquifer rocks crop out in the northwestern half of the quadrangle. Here the bottom of the aquifer can often be approximately defined by picks from drillers' logs of the "top" of the Glen Rose and/or Walnut Formation. This pick is made at the first descriptions of "blue lime." As described by water well drillers this occurrence probably varies considerably stratigraphically. The "blue lime" usually occurs in the Walnut or in the upper Glen Rose.

Fig. 2 includes a geologic cross section approximately perpendicular to the local strike of the Balcones fault zone. This section shows the Edwards Aquifer to exist in a series of discrete blocks with the aquifer more or less continuous between blocks and usually successively lower in elevation toward the southeast. The highest and lowest recorded water levels in this quadrangle are shown approximately on the section.

ACKNOWLEDGEMENTS

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THE PARACNEMID GLAND OF *BUFO ALVARIUS*

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ABSTRACT

The gross and microscopic anatomy of the venom-producing paracnemid gland of *Bufo alvarius* was studied by light and electron microscopy. The paracnemid gland, composed of numerous simple acinar glands each have a duct opening onto the skin. Each acinar gland is a unit with a lumen surrounded by a double cell layer. The outer layer resembles myoepithelial cells and contains filaments, mitochondria, granular ER, ribosomes and occasional pinocytotic vesicles. An intercellular (intervillous) space separates the outer and inner layers. The inner layer contains some granular and agranular ER, membrane-bounded particles, vacuoles and abundant mitochondria. The inner layer appears continuous with the secretory product. The cell layers and secretory product give negative chromaffin and Schultz reactions. The intercellular space contains periodic acid-Schiff (PAS) positive material, while the layers are negative; the secretory product is moderately PAS positive. The outer layer may be contractile and possibly assists in discharge of the secretory product. Both layers may participate in venom synthesis. Perhaps because of its caudal location, the paracnemid gland need not be as efficient as the anteriorly-situated parotoid glands.

INTRODUCTION

Bufo alvarius is the toad that appears to be richest in poison-producing glands (Lutz, 1971). In addition to small granular glands dispersed throughout the integument and 2 large parotoid glands on the shoulders, accessory glands, named paracnemid glands, occur on the limbs.

In a previous study (Cannon and Hostetler, 1976) we reported the anatomy and histochemistry of the parotoid gland in *B. alvarius*. Much is known regarding

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the biochemical (Chen and Chen, 1933; Daly and Witkop, 1966; Chen and Kovarikova, 1967; Yoshida, *et al.*, 1976) and pharmacological (Okada, 1966; Chen and Kovarikova, 1967; Deulofeu and Ruveda, 1971; Meyer and Linde, 1971) characteristics of the venoms produced by this gland. In contrast, virtually nothing is known about the morphology and histochemistry of the paracnemid gland in Bufonidae, although there are considerable data regarding the skin glands of other amphibia. These glands include the mucous and poison-producing granular glands of *Rana sylvatica* (Helf and Stark, 1941) and *R. pipiens* (Dapson, 1970; Dapson, *et al.*, 1973), and the various integumental glands of species of the genera *Bufo, Scaphiopus, Hyla, Microhyla (Gastrophryne), Pipa, Eleutherodactylus* (Elias and Shapiro, 1957), *Phyllomedusa* (Blaylock, *et al.*, 1976) and *Triturus* (Bani, 1969). The skin glands occurring in numerous fishes and other amphibians have also been reviewed (Quay, 1972).

This paper presents the detailed anatomy of the paracnemid gland of *B. alvarius* at both the light and electron microscopic levels, and includes histochemical findings.

MATERIALS AND METHODS

Adult Colorado River toads (*B. alvarius*), newly captured, were obtained from Hermosa Reptile, Inc. (Hermosa Beach, California) and from Mr. C.D. Sullivan (Nashville, Tennessee). During the investigation each animal was retained approximately 60 days. Animals were fed twice each week, at 3-day intervals, on Australian brooder crickets (Selph's Cricket Ranch, Desoto Station, Tennessee). No animal was force-fed, but allowed to eat until full.

To avoid the skin gland secretion frequently occasioned during chemical anesthesia, the animals were cooled at 4 C until inactive. The paracnemid glands located on the dorsal aspect of the hind limbs were excised, *in toto*. For light microscopy, the glands were fixed in 10% neutral formalin, Zenker-formol solution (Preece, 1965) or 80% ethyl alcohol and embedded in butoxyethanol-glycol methacrylate (Ruddell, 1967). Sections (4 μm) were cut on the JB-4A Sorvall microtome (Du pont, Wilmington, Delaware) and stained with hematoxylin and eosin, or treated with periodic acid-Schiff (PAS) reagents and counterstained with Harris hematoxylin. Paracnemid tissue also was subjected to the chromaffin reaction in which frozen sections were examined for the presence of 5-hydroxytryptamine or related compounds (Barka and Anderson, 1963). The steroid nucleus, indicating the presence of cholesterol or its esters was determined from 20 μm frozen sections using a modified Schultz method (Weber, *et al.*, 1956).

For electron microscopy, paracnemid glands were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 hr at room temperature. The glands then were rinsed in 2 changes of buffer, post-fixed in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.2) for 1 hr and prestained in saturated aqueous uranyl acetate

for 30 min. The tissue was dehydrated in a graded ethanolic series and placed in propylene oxide. The glands were then embedded in increasing concentrations of Epon 812 (Luft, 1961). Thin sections were cut on the LKB Ultratome III, post-stained with uranyl acetate and lead citrate and examined on a Hitachi HU-12 electron microscope.

RESULTS

In *B. alvarius* the paracnemid glands on the hind limbs (Fig. 1) each measure approximately 1 cm by 0.5 cm and lie under the skin and subcutaneous connective tissue. No nerve fibers are seen under the basement membrane. A paracnemid gland consists of many individual 2 X 2 mm, simple acinar (alveolar) glands (Fig. 2) (Elias and Shapiro, 1957), each surrounded by connective tissue and having a duct that opens onto the skin. At collection and during the study the paracnemid glands contained the thick secretory product.

Each acinar gland is a unit with a lumen (Fig. 2) surrounded by a double cell layer (Figs. 3,4). The exterior surface of the cells composing the outer layer rests on a thin continuous basement membrane, while the interior surface forms short microvilli which either interdigitate with microvilli formed by the outer plasmalemma of the inner cell layer (Figs. 4,7) or project into an intercellular (intervillous) space. Infrequently, desmosomes are seen at points of contact between the outer and inner cell layers (Fig. 7). The periodic acid-Schiff (PAS) reaction demonstrates a strongly positive product in the area of the basement membrane. The intercellular (intervillous) space between the outer and inner cell layers contains PAS positive material, while the layers, themselves, are negative (Fig. 3).

Cells forming the outer layers are elongated, continuous with one another and desmosomes may be present at their points of contact (Fig. 6). Moderate numbers of filaments reside in the cytoplasm and often are continuous with the desmosomes (Fig. 6). The elongated nuclei are rarely segmented; the chromatin varies from finely granular (Figs. 4,5) to dark with some clumping and nucleoli are occasionally seen. Some round to rod-shaped mitochondria are observed, often in close association with the granular ER (Fig. 5). The latter usually occurs in scant amounts throughout the outer layer cells, but occasionally moderate quantities of granular ER are observed which may be in close relationship to one or more mitochondria (Fig. 5). In addition, polyribosomes are sometimes observed in areas of increased granular ER. Ribosomes dot the nuclear envelope (Fig. 5), while agranular ER and Golgi complexes are rarely seen. Occasional pinocytotic vesicles are present on the exterior surface of the outer cell layer. The chromaffin and Schultz reactions are negative for the outer cell layer. Melanophores between the epidermis and dermis show a positive chromaffin reaction.

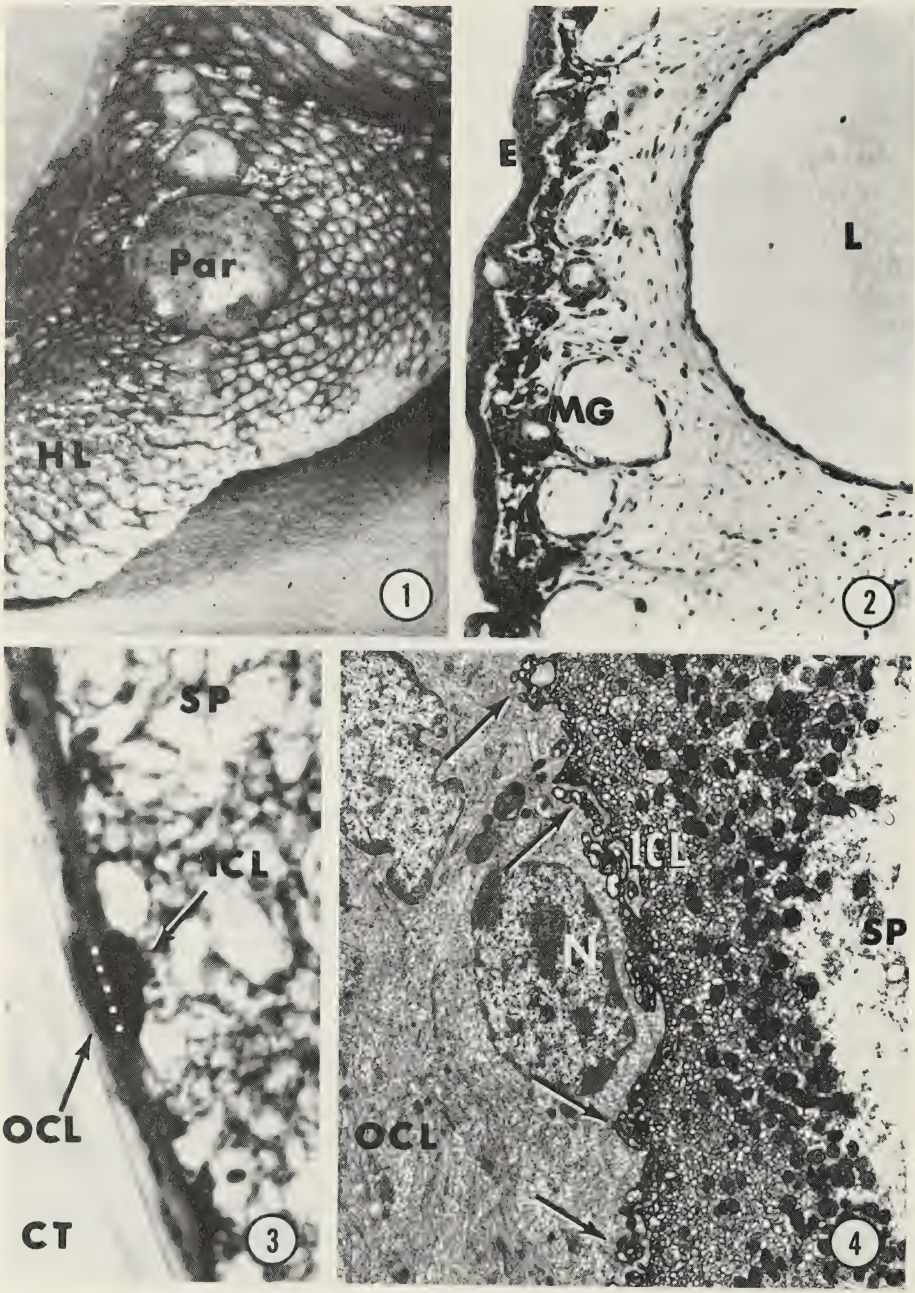

Plate 1.

(Plate 1 Continued)

Legend:
CT, Connective Tissue
E, Epidermis
HL, Hind Limb
ICL, Inner Cell Layer
L, Acinar (alveolar) gland
MG, Mucous Gland
N, Nucleus
OCL, Outer Cell Layer
Par, Paracnemid gland
SP, Secretory Product
Arrows, Microvilli interdigitations
Dots, Intercellular space containing PAS positive material

Figure 1. A paracnemid gland (Par) and the hind limb (HL) of *B. alvarius* is indicated.

Figure 2. A paracnemid gland consists of many individual acinar (alveolar) glands (L). The epidermis (E) and subcutaneous tissue containing skin mucous glands (MG) are seen. Hematoxylin and eosin. (Light micrograph). X100.

Figure 3. Each acinar gland is a unit surrounded by outer (OCL) and inner (ICL) cell layers. Note the subcutaneous connective tissue (CT) and secretory product (SP) within the lumen of the gland. The dotted line indicates PAS positive material within the intercellular space. Periodic acid–Schiff reaction. (Light micrograph). X1,000.

Figure 4. Microvilli interdigitations (arrows) are seen between the outer (OCL) and inner (ICL) cell layers. A nucleus (N) of an outer layer cell exhibits finely granular chromatin and a nucleolus might be present. Abundant mitochondria are present in the inner margin of the inner cell layer and continue into the secretory product (SP). (Electron micrograph). X7,000.

The outer plasmalemma of the cells composing the inner layer frequently forms microvilli that interdigitate with the microvilli of the outer cell layer (Figs. 4,7). In contrast, that portion of the inner cell layer toward the center of a gland is devoid of a plasmalemma and its cytoplasm is continuous with the secretory product (Fig. 4). Furthermore, cell boundaries between inner layer cells are indistinct and it appears that not every cell possesses a nucleus. Those nuclei present are large, elongated, and possess dark moderately-clumped chromatin (Fig. 7). Nucleoli are observed occasionally. Granular and agranular ER is seen throughout the cytoplasm (Fig. 8). Membrane-bounded particles, possibly lysosomes, are also observed in Figure 8. Abundant mitochondria, usually in the inner margin of the cells, are present (Figs. 4,7). In addition, occasionally vacuoles, some containing finely granular material, are seen primarily in the inner glandular portion of the cytoplasm. Golgi complexes are rarely observed. The nearly homogenous secretory product demonstrates localized regions of vacuoles and granular material. The inner cell layer and the secretory product are negative for both the chromaffin and Schultz reactions. The secretory product is moderately PAS positive.

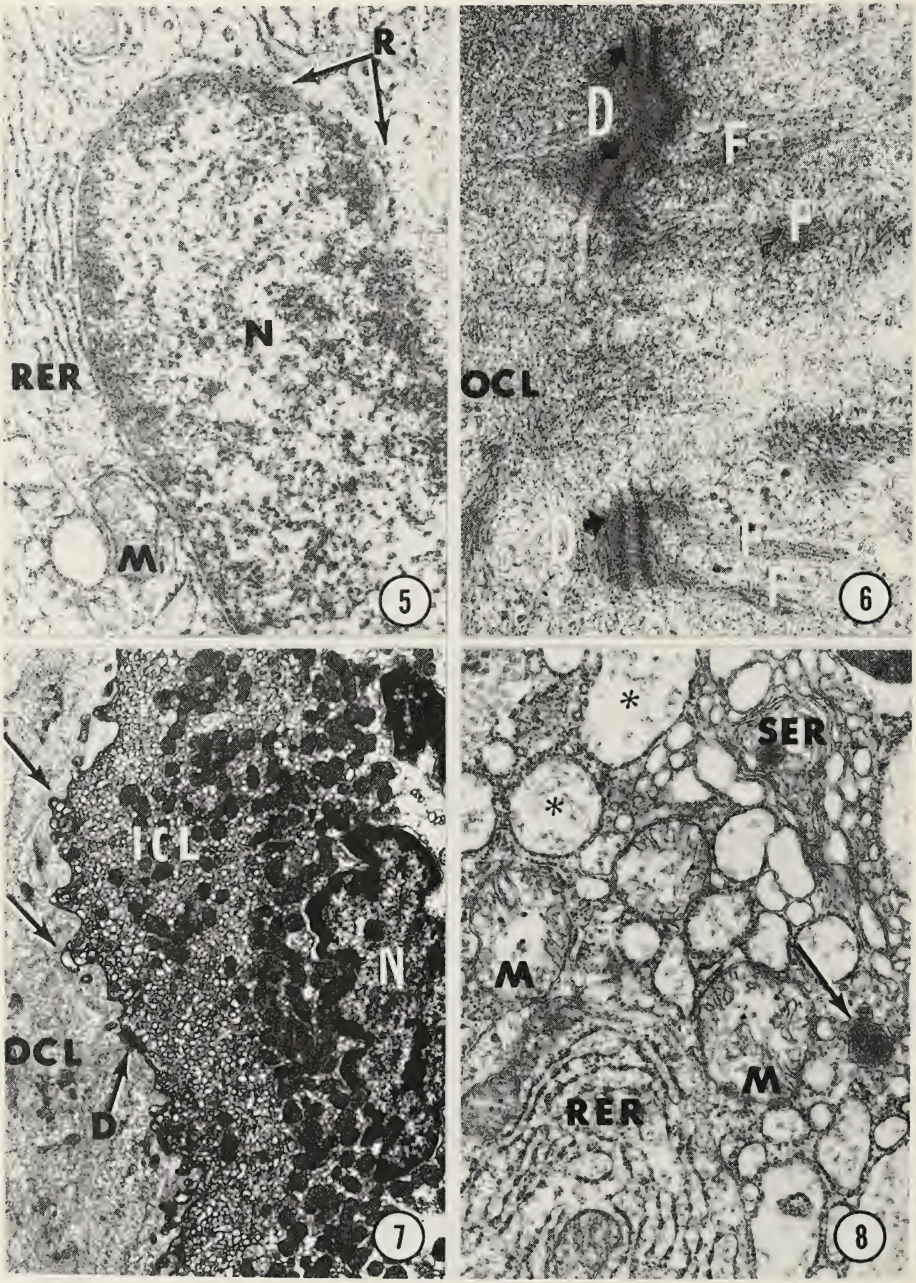

Plate 2.

(Plate 2 Continued)

Legend:
D, Desmosomes (s)
F, Filaments
ICL, Inner Cell Layer
M, Mitochondria
N, Nucleus
OCL, Outer Cell Layer
R, Ribosomes
RER, Granular Endoplasmic Reticulum
SER, Agranular Endoplasmic Reticulum
Arrows, Microvilli interdigitations
Arrow, Lysosome
Asterisks, Possible degenerating mitochondria

Figure 5. In outer layer cells, ribosomes (R) dot the nuclear envelope, while moderate amounts of granular endoplasmic reticulum (RER) are present. Note the mitochondrion (M) and granular chromatin of the nucleus. (Electron micrograph). X22,000.

Figure 6. Outer layer cells (OCL) are contiguous with one another and desmosomes (D) may be present at their points of contact. Filaments (F) occur in the cytoplasm and often are continuous with the desmosomes. (Electron micrograph). X56,000.

Figure 7. Desmosomes (D) are occasionally present at points of contact between outer (OCL) and inner (ICL) cell layers. Nuclei (N) of inner layer cells are elongated and demonstrate dark to moderately-clumped chromatin. These nuclei are released as part of the secretory product. Note the numerous mitochondria in the inner layer cell and the microvilli interdigitations (arrows) between layers. (Electron micrograph). X7,000.

Figure 8. Inner layer cells contain granular (RER) and agranular (SER) endoplasmic reticulum. A possible lysosome (arrow), mitochondria (M) and probably degenerating mitochondria (asterisks) are seen. (Electron micrograph). X21,000.

DISCUSSION

The inner cell layer of the paracnemid gland is involved in synthesis as indicated by the presence of abundant mitochondria, granular and agranular reticulum, vacuoles and secretory product within the cytoplasm. Moreover, this layer appears to undergo an apocrine type of secretion like that of the parotoid gland in *B. alvarius* (Cannon and Hostetler, 1976) and *B. marinus* (Hostetler and Cannon, 1974).

The paracnemid gland, like the granular integumentary glands of *R. pipiens* (Noble and Noble, 1944), consists of 2 cell layers and disintegrating nuclear material from the inner layer contributes to the secretory product. In an earlier description of granular skin glands in *R. sylvatica*, Helf and Stark (1941) suggested that secretion granules were remnants of degenerating cells (particularly nucleoli), and Dawson (1937) proposed that material passed from the nucleus

layer cells of the paracnemid gland resemble the inner layer of the parotoid gland in *B. alvarius* (Cannon and Hostetler, 1976) and *B. marinus* (Hostetler and Cannon, 1974), since all 3 glands lack distinct boundaries between inner layer cells.

A negative chromaffin reaction within the inner and outer cell layers and in the secretory product of the paracnemid gland indicates the absence of 5-hydroxytryptamine or analogous compounds, known to be present in the parotoid gland of *B. alvarius* (Erspamer, *et al.*, 1967; Deulofeu and Ruveda, 1971; Cannon and Hostetler, 1976). Furthermore, 5-hydroxytryptamine occurs in the skin of other anurans, including, *B. regularus* (Kramer, 1970), *R. esculenta* (Vialli and Quaroni, 1956), *Xenopus* (Vanable, 1964; Kramer, 1970), and in the genera *Discoglossus* and *Bombina* Vialli and Quaroni, 1956). However, adrenaline, noradrenaline, dopamine and N-methyldopamine have not been demonstrated in skin glands from *B. alvarius* (Chen and Kovaříková, 1967; Deulofeu and Ruveda, 1971), but are present in the parotoid gland and venoms of *B. marinus* (Chen and Kovaříková, 1967; Deulofeu and Ruveda, 1971; Hostetler and Cannon, 1974). And although the luminal skin glands of *R. pipiens* contain dopamine they, like the paracnemid gland, lack 5-hydroxytryptamine (Dapson, *et al.*, 1973).

The paracnemid gland, like the parotoid gland and venoms from *B. marinus* (Hostetler and Cannon, 1974) and the luminal skin glands of *R. pipiens* (Dapson, *et al.*, 1973), is negative for steroids. These results show steroids are not present, however, such materials may be bound in a non-reactive product or occur in an insufficient concentration to be detected. In contrast, the presence of steroids is demonstrated in the parotoid gland and secretory product of *B. alvarius* (Cannon and Hostetler, 1976) as well as in the skin of other anurans (Chen and Chen, 1933; Michl and Kaiser, 1963; Albuquerque, *et al.*, 1971).

Mucous glands of amphibia are nearly always strongly PAS positive (Dawson, 1920; Noble and Noble, 1944; Pedrazzi, 1955; Burgers and van Oordt, 1956; Quay, 1972). Conversely, granular glands (and their secretions) of amphibian integument tend to demonstrate little or no PAS reactivity (Pedrazzi, 1955; Quay, 1972). Exceptions to this seem to be the granular cellular glands of *R. pipiens* (Dapson, *et al.*, 1973) and the paracnemid gland of the present study. The paracnemid, like the parotoid gland of *B. marinus* (Hostetler and Cannon, 1974), demonstrates an intercellular (intervillous) space containing PAS positive material, between the outer and inner cell layers. In comparison, no intercellular space is seen in the parotoid gland of *B. alvarius* (Cannon and Hostetler, 1976).

The outer cell layer of the paracnemid gland, because of its orientation and the presence of cytoplasmic filaments and some pinocytotic vesicles resembles myoepithelial or smooth muscle cells (Ellis, 1965). Possible contraction of this layer may assist in discharge of the secretory product. Furthermore, in the parotoid gland of *B. alvarius* pinocytotic vesicles occur in both the outer and

inner cell layers (Cannon and Hostetler, 1976), but only in the outer layer of the paracnemid gland. Still, the presence of occasional pinocytotic vesicles in the paracnemid gland suggests a way that materials may enter the outer cells and be transported via micropinocytosis (Holter, 1960; Palade and Bruns, 1968). The possibility of active transport is further enhanced by the occurrence of mitochondria.

The paracnemid gland of *B. alvarius* is morphologically similar to the parotoid gland in this animal and in *B. marinus*, and resembles the granular integumentary glands of several other anurans. Histochemically, the paracnemid gland more closely compares with the parotoid gland of *B. marinus* than with the parotoid of *B. alvarius*. Perhaps because of its caudal location it need not be as efficient as the anteriorly-situated parotoid glands. Further investigation may disclose an as yet unsuspected function of the paracnemid gland.

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A CHECKLIST OF TEXAS LICHENS

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ABSTRACT

A checklist of the lichen flora of Texas has been compiled recognizing 492 species in 101 genera distributed in 35 families. A separate list of 329 synonyms and excluded names is included. The list is based on literature reports from over 100 sources published between 1847 and 1977. Maps showing the distribution of lichen taxa reported from Texas counties are also included.

INTRODUCTION

In 1847 Edward Tuckerman a resident of Amherst, Massachusetts, published the first record of a Texas lichen (*Pyxine sorediata*). This was only one of numerous Texas lichen taxa Tuckerman reported between 1847 and 1888 based on the diligent field work of several early naturalists, especially Charles C. Wright. Wright came to Texas in 1837 and can be credited with making most of the important early lichen collections from the state. Wright accompanied the Mexican Boundary Survey in 1849 and spent much of the winter of 1850 in San Antonio collecting lichens in central Texas (Geiser, 1948). Wright's Texas lichen specimens, presently housed in the Farlow Herbarium of Harvard University, were studied by Tuckerman, and many of these collections were described as new species (Tuckerman, 1882, 1888). Other 19th century Texas lichen collectors of special note include Henry William Ravenel, Elihu Hall, and Jacob Boll. Boll's collections from the Dallas region were sent to Europe, and several species were reported in a small paper by Müller Argoviensis (1877). Included in this paper was the description of the now well-known *Parmelia bolliana*. Most of Hall's and Ravenel's collections were from the Gulf Coast areas and were included in Tuckerman's *Synopsis of the North American Lichens* (1882, 1888).

Although many lichen taxa were reported from Texas between 1847 and 1888, few additional species were added to the flora until the 1930's. The studies of Whitehouse (1933, 1934) concentrated on areas around El Paso, Austin, Bastrop, and Llano County. Fink (1935) included records for over 130 taxa listing only

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"Texas" in his *Lichen Flora of the United States*, and Steyermark and Moore (1933) added several reports of species from extreme western Texas.

Figure 1 indicates chronologically how our knowledge of the Texas lichen flora up to 1970 was accumulated. The cumulative number of reported taxa at the end of each 10-year period is expressed as a percentage of the total number of taxa reported by 1970. Texas is compared with other western states which have had lichen catalogs published recently: Arizona (Nash & Johnsen, 1975), Colorado (Shushan & Anderson, 1969), New Mexico (Egan, 1972). The efforts of early naturalists such as Charles Wright made information on the Texas lichen flora available much sooner than these other western states.

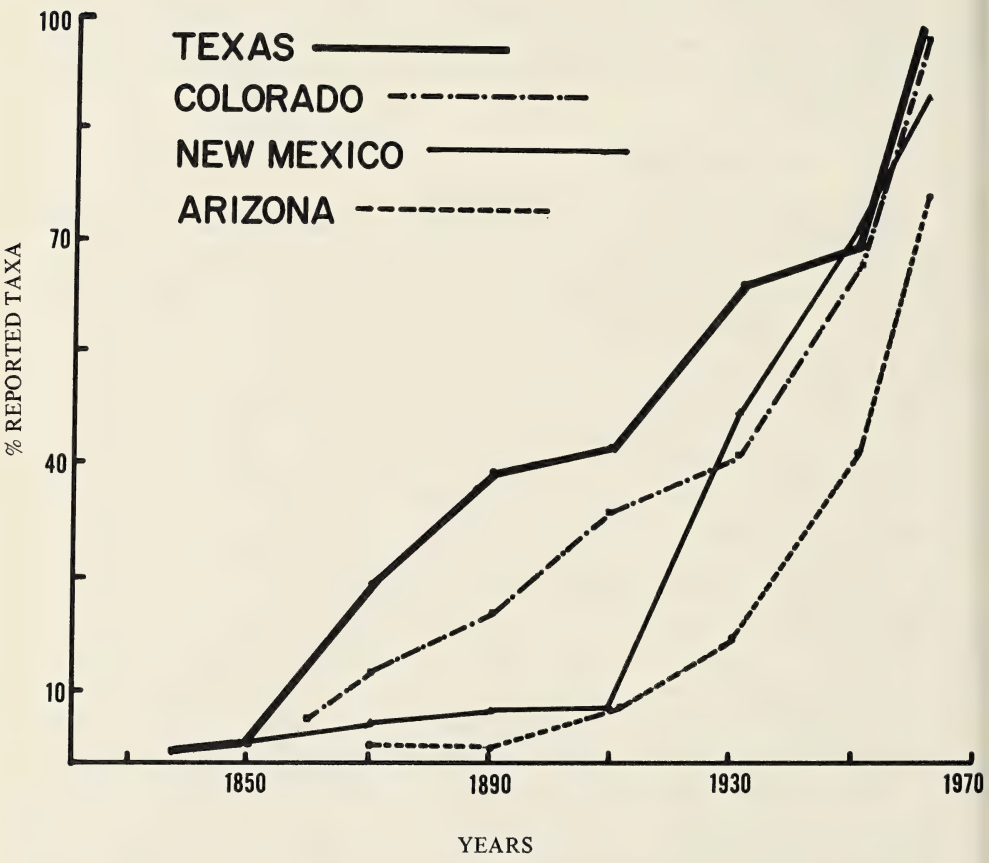

Figure 1. Chronological accumulation of our knowledge of the lichen floras of 4 western states. Data for Colorado from Shushan & Anderson (1969), Arizona from Nash & Johnsen (1975), and New Mexico from Egan (1972).

Several professional lichenologists have made extensive collections of Texas lichens in recent years, although little material is housed in herbaria within the state. Mason Hale of the Smithsonian Institution collected throughout the eastern portions of the state in 1955, and Clifford Wetmore of the University of Minnesota has published on the macrolichens of Big Bend National Park (Wetmore, 1976). Several new additions to the lichen flora of Texas were also added by Egan (1976, 1977). Other lichenologists who have done extensive collecting in Texas include W.A. Weber and S. Shushan of the University of Colorado, R.A. Anderson of the University of Denver, and S. Tucker from Louisiana State University. Specimens collected by these individuals and others have been listed in the literature from the following major herbaria: BM, BPI, CANL, COLO, DUKE, F, FH, G, H, L, LSU, MICH, MIN, MO, MSC, NY, PH, REN, S, TAES, TEX, TSN, UPS, US, W, WIS, the Biology Department Herbarium at Texas A&M University (TAMU) and several private collections.

Although the specimens collected by these individuals have been reported in many publications, there has never been a comprehensive treatment of the Texas lichens or any compilation of the status of our knowledge of the lichens of the state. It is hoped that this paper will form a starting point for future floristic work on Texas lichens and will assist curators holding Texas lichen specimens to update names on Texas material. Interested individuals are encouraged to correspond with the author about unknown lichen collections from Texas or about material in need of revision. Our knowledge of the distribution of Texas lichen taxa is still very incomplete, and many areas of the state remain lichenologically unexplored.

DISTRIBUTION OF TEXAS LICHENS

Lichen taxa have been listed in the literature from only 91 of the 254 counties in the state. The greatest number of lichen species (120) has been reported from Brewster County. Other counties with numerous reported lichen taxa include Sabine (50), Nacogdoches (39), and Harris (25). Figure 2 shows the distribution of county records. Figure 2A indicates all counties with at least one reported lichen species. Figures 2B-D indicate counties which have had 5 or more taxa reported, 10 or more taxa reported, and 20 or more taxa reported, respectively. A considerable collecting effort is still required before general distribution patterns for Texas lichen taxa can be characterized with any certainty.

CHECKLIST OF LICHENS REPORTED FROM TEXAS

The following lists of lichen taxa are based on published literature reports from more than 100 sources from 1847 to 1977. Although an attempt has been made to make this literature survey exhaustive, there are undoubtedly publications that have been missed. Only species which are specifically cited for Texas

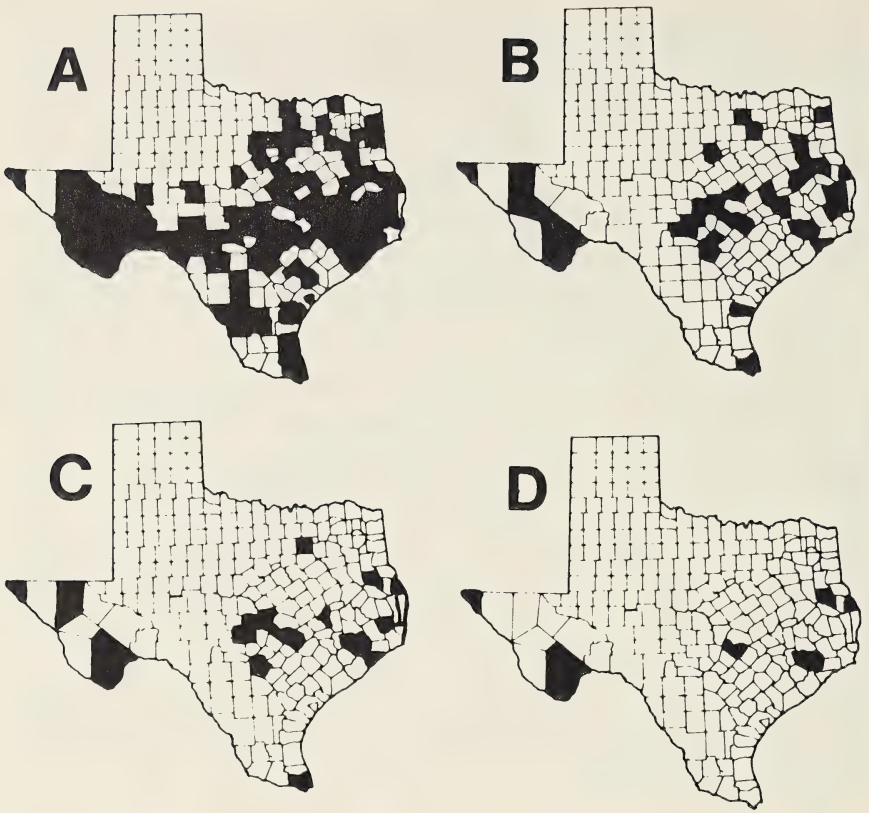

Figure 2. Distribution by county of reported lichen taxa from Texas. (A) indicates all counties from which there is at least 1 lichen species reported, (B) 5 or more species, (C) 10 or more species, (D) 20 or more taxa reported.

have been included, and early reports of Wright's collections from the "Organ Mountains, Texas" have been excluded since these mountains lie wholly within the state of New Mexico (Egan, 1972). As a special aid to students and researchers using older collections and publications, the checklist has been divided into 3 parts: (1) a systematic synopsis of the Texas lichen genera by family, (2) the presently accepted lichen taxa for the state of Texas, and (3) the synonyms and other excluded names which have been used previously in publications concerning Texas lichens. Subspecific taxa have been listed for Texas specimens, but they have been generally excluded from this list. Older reports of subspecific taxa are included in the list of synonyms and excluded names since many of these taxa are now recognized at the specific level. This list also contains several taxa for which the presently accepted name is uncertain. The proper disposition of these names will be made in a future edition. Nomenclature generally follows Hale and Culberson (1970) and Hale (1974b, c, d, e) for the segregate parmeliaceous genera. A separate list taxa with their type locality in Texas is being prepared and will be published at a later date.

This checklist was compiled from an extensive card file which includes (if available) for each taxon reported the collection localities in Texas, collector(s), collection numbers, collection dates, author-date-page number in the literature, herbarium in which specimens are deposited, and notation of type status. This information has been omitted from the present "barebones" checklist, because its inclusion would have made the manuscript much too long. If those using this paper would like these specific references to taxa or other information concerning the basis for the inclusion or exclusion of a particular taxon from the lists, that information will be forwarded on request. All published information on Texas lichens (including literature references, page numbers, localities, synonyms, herbaria, collectors, collection dates, etc...) is being entered into a computerized data base. Once this data base is complete, retrieval of published information on Texas lichens will be available on request in many formats.

SYSTEMATIC SYNOPSIS OF THE GENERA

The following systematic synopsis of the Texas lichen genera is based on a synthesis of recently revised systems of lichen classification (Poelt, 1973; Henssen & Jahns, 1974; Hale, 1974a). Concepts of lichen families and orders are still in a state of flux, and the usual arrangement of families in the order Lecanorales into suborders has been omitted until there is greater agreement as to the appropriate limits for the suborders. The system presented below will surely be revised as these higher taxonomic categories are aligned into more "natural" groups. The numbers in parentheses indicate the number of recognized species from Texas included in each genus.

CLASS: ASCOMYCETES

SUBCLASS: ASCOMYCETIDAE

Order Sphaeriales
- Porinaceae
  - *Belonia* (1)
  - *Porina* (2)
- Pyrenulaceae
  - *Anthracothecium* (1)
  - *Melanotheca* (2)
  - *Parmentaria* (2)
  - *Polyblastiopsis* (2)
  - *Pyrenula* (3)
  - *Trypethelium* (4)
- Strigulaceae
  - *Strigula* (2)
- Verrucariaceae
  - *Dermatocarpon* (7)
  - *Endocarpon* (1)
  - *Verrucaria* (2)

Order Caliciales
- Caliciaceae
  - *Calicium* (1)
  - *Chaenotheca* (1)
- Mycocaliciaceae
  - *Mycocalicium* (1)

Order Lecanorales
- Collemataceae
  - *Collema* (15)
  - *Leptogium* (18)
- Parmeliaceae
  - *Bulbothrix* (3)
  - *Cetraria* (3)
  - *Hypotrachyna* (4)
  - *Parmelia* (15)
  - *Parmelina* (5)
  - *Parmeliopsis* (2)
  - *Parmotrema* (25)
  - *Pseudevernia* (1)
  - *Pseudoparmelia* (7)
  - *Xanthoparmelia* (17)
- Anziaceae
  - *Anzia* (2)
- Lecanoraceae
  - *Aspicilia* (3)

Haematomma (2)
Lecanora (15)
Lecideaceae
Bacidia (5)
Bombyliospora (2)
Lecidea (15)
Lopadium (3)
Rhizocarpon (1)
Candelariaceae
Candelaria (2)
Candelariella (2)
Candelina (2)
Cladoniaceae
Cladina (2)
Cladonia (27)
Umbilicariaceae
Lasallia (1)
Umbilicaria (1)
Ramalinaceae
Ramalina (15)
Usneaceae
Usnea (15)
Acarosporaceae
Acarospora (19)
Sarcogyne (3)
Pannariaceae
Pannaria (4)
Parmeliella (1)
Coccocarpiaceae
Coccocarpia (4)
Heppiaceae
Heppia (1)
Peltula (9)
Peltigeraceae
Peltigera (5)
Nephromataceae
Nephroma (1)
Stictaceae
Lobaria (2)
Pseudocyphellaria (1)
Sticta (1)
Lichinaceae
Pyrenopsis (1)
Rechingeria (1)
Synalissa (2)
Thyrea (3)
Teloschistaceae
Caloplaca (14)
Teloschistes (3)
Xanthoria (6)
Physciaceae
Anaptychia (1)
Buellia (12)
Dimelaena (1)
Dirinaria (8)
Heterodermia (11)
Physcia (25)
Physciopsis (2)
Physconia (2)
Pyxine (3)
Rinodina (3)
Pertusariaceae
Ochrolechia (3)
Pertusaria (11)
Phlyctis (1)

Order Ostropales

Thelotremataceae
Diploschistes (1)
Leptotrema (6)
Ocellularia (4)
Thelotrema (3)
Graphidaceae
Glyphis (2)
Graphina (2)
Graphis (7)
Gyrostomum (1)
Medusulina (1)
Melaspilea (1)
Phaeographis (2)
Sarcographa (1)
Gyalectaceae
Coenogonium (3)

SUBCLASS: LOCULOASCOMYCETIDAE

Order Arthoniales

Arthoniaceae
Arthonia (15)
Arthothelium (2)
Lecanactidaceae
Schismatomma (1)
Opegraphaceae
Chiodecton (3)
Opegrapha (3)
Roccellaceae
Roccella (1)

Order Dothideales

Pleosporaceae
Arthopyrenia (2)
Microthelia (1)

CLASS: FUNGI IMPERFECTI

Lepraria (2)
Normandina (1)

UNCERTAIN POSITION

Speerschneidera (1)

LIST OF GENERA AND SPECIES[1]

Acarospora Mass.

albida Magn.
bella (Nyl.) Jatta
caesiofusca (Müll. Arg.) Magn.
chlorophana (Wahlenb. *ex* Ach.) Mass.
chrysops (Tuck.) Magn.
citrina (Tayl.) Zahlbr.
contigua Magn.
epilutescens Zahlbr.
glaucocarpa (Wahlenb. *ex* Ach.) Körb.
macrospora (Hepp) Bagl.
rubicunda Magn.
schleicheri (Ach.) Mass.
sinoptica (Wahlenb. *ex* Ach.) Körb.
socialis Magn.
subalbida Magn.
subcontigua Magn.
tenebrica Magn.
texana Magn.
xanthophana (Nyl.) Jatta

Anaptychia Körb.

palmulata (Michx.) Vain.

Anthracothecium Hampe *ex* Mass.

pyrenuloides (Mont.) Müll. Arg.

Anzia Stizenb.

colpodes (Ach.) Stizenb.
ornata (Zahlbr.) Asah.

Arthonia Ach.

atrata (Fée) Müll. Arg.
caribaea (Ach.) Mass.
chiodectella Nyl.
cinnabarina (DC.) Wallr.
conferta (Fée) Nyl.
erubescens Will.
erupta Nyl.
lecideella Nyl.
leucastraea Tuck.
microspermella Will.
pyrrhuliza Nyl.
radiata (Pers.) Ach.
ravenelii Tuck.
rubella (Fée) Nyl.
viridicans Will.

Arthopyrenia Mass.

cinchonae (Ach.) Müll. Arg.
subprostans (Nyl.) Müll. Arg.

Arthothelium Mass.

interveniens (Nyl.) Zahlbr.
macrothecum (Fée) Mass.

Aspicilia Mass.

calcarea (L.) Mudd
cinerea (L.) Körb.
contorta (Hoffm.) Kremp.

Bacidia De Not.

incompta (Borr. *ex* Hook.) Anzi
medialis (Tuck.) Zahlbr.
schweinitzii (Tuck.) Schneid.
stigmatella (Tuck.) Zahlbr.
suffusa (Fr.) Schneid.

Belonia Körb.

americana Fink

Bombyliospora De Not.

pachycheila (Tuck.) Zahlbr.
tuberculosa (Fée) Mass.

Buellia De Not.

alboatra (Hoffm.) Branth & Rostr.
callispora (Knight.) J. Stein.
caloosensis Tuck.
curtisii (Tuck.) Imsh.
disciformis (Fr.) Mudd
glaziouana (Kremp.) Müll. Arg.
lauri-cassiae (Fée) Müll. Arg.
lepidastra (Tuck.) Tuck.
punctata (Hoffm.) Mass.
retrovertens Tuck.
spuria (Schaer.) Anzi
stillingiana J. Stein.

Bulbothrix Hale

confoederata (W. Culb.) Hale
coronata (Fée) Hale
goebelii (Zenker *in* Goebel & Kunze) Hale

Calicium Pers.

subtile Pers.

[1]Names in brackets are species (or taxa) which should be included in the genus indicated but for which the appropriate taxonomic combination has never been published. In some cases, especially in the list of Synonyms and Excluded Names, taxa in brackets are names which have been cited in the literature for Texas lichens but for which I have been unable to locate any reference to an original publication source.

Caloplaca Th. Fr.

camptidia (Tuck.) Zahlbr.
cerina (Ehrh.) Th. Fr.
cinnabarina (Ach.) Zahlbr.
cirrochroa (Ach.) Th. Fr.
[*Lecanora diphasia* Tuck.]
eugyra (Tuck.) Zahlbr.
ferruginea (Huds.) Th. Fr.
flavorubescens (Huds.) Laundon
flavovirescens (Wulf.) Dalla Torre & Sarnth.
[*Lecanora floridana* Tuck.]
holocarpa (Hoffm.) Wade
microphyllina (Tuck.) Hasse
pollinii (Mass.) Jatta
wrightii (Will. *ex* Tuck.) Fink

Candelaria Mass.

concolor (Dicks.) B. Stein
fibrosa (Fr.) Müll. Arg.

Candelariella Müll. Arg.

aurella (Hoffm.) Zahlbr.
vitellina (Ehrh.) Müll. Arg.

Candelina Poelt

mexicana (B. de Lesd.) Poelt
submexicana (B. de Lesd.) Poelt

Cetraria Ach.

coralligera (W. Web.) Hale
fendleri (Nyl.) Tuck.
viridis Schwein.

Chaenotheca (Th. Fr.) Th. Fr.

trichialis (Ach.) Th. Fr.

Chiodecton Ach.

montagnaei Tuck.
perplexum Nyl.
sanguineum (Sw.) Vain.

Cladina (Nyl.) Harm.

evansii (Abb.) Hale & W. Culb.
subtenuis (Abb.) Hale & W. Culb.

Cladonia Wigg.

bacillaris (Ach.) Nyl.
balfourii Cromb.
beaumontii (Tuck.) Vain.
caespiticia (Pers.) Flörke
capitata (Michx.) Spreng.
cariosa (Ach.) Spreng.
chlorophaea (Flörke *ex* Somm.) Spreng.
clavulifera Vain.
cristatella Tuck.
cryptochlorophaea Asah.
didyma (Fée) Vain.
fimbriata (L.) Fr.
furcata (Huds.) Schrad.
grayi Merr. *ex* Sandst.
leporina Fr.
macilenta Hoffm.
pleurota (Flörke) Schaer.
polycarpoides Nyl.
pyxidata (L.) Hoffm.
ravenelii Tuck.
santensis Tuck.
squamosa (Scop.) Hoffm.
strepsilis (Ach.) Vain.
subcariosa Nyl.
symphycarpa (Ach.) Fr.
uncialis (L.) Wigg.
verticillata (Hoffm.) Schaer.

Coccocarpia Pers.

cronia (Tuck.) Vain.
erythroxili (Spreng.) Swinsc. & Krog
incisa Pers.
pellita (Ach.) Müll. Arg.

Coenogonium Ehrenb.

implexum Nyl.
interplexum Nyl.
interpositum Nyl.

Collema Wigg.

callibotrys Tuck.
coccophorum Tuck.
conglomeratum Hoffm.
cristatum (L.) Wigg.
fasciculare (L.) Wigg.
flaccidum (Ach.) Ach.
furfuraceum (Arn.) Du Rietz
leptaleum Tuck.
leucopeplum (Tuck.) Schneid.
nigrescens (Huds.) DC.
polycarpon Hoffm.
subfurvum (Müll. Arg.) Degel.
tenax (Sw.) Ach.
texanum Tuck.
tuniforme (Ach.) Ach.

Dermatocarpon Eschw.

fluviatile (G. Web.) Th. Fr.
hepaticum (Ach.) Th. Fr.
lachneum (Ach.) A.L. Sm.
miniatum (L.) Mann
moulinsii (Mont.) Zahlbr.
rufescens (Ach.) Th. Fr.
tuckermanii (Rav.) Zahlbr.

Dimelaena Norm.

oreina (Ach.) Norm.

Diploschistes Norm.

scruposus (Schreb.) Norm.

Dirinaria (Tuck.) Clem.

applanata (Fée) Awas.
aspera (Magn.) Awas.
confluens (Fr.) Awas.
confusa Awas.
frostii (Tuck.) Hale & W. Culb.
leopoldii (Stein) Awas.
picta (Sw.) Clem. & Schear.
purpurascens (Vain.) B. Moore

Endocarpon Hedw.

pusillum Hedw.

Glyphis Ach.

achariana Tuck.
cicatricosa Ach.

Graphina Müll. Arg.

acharii (Fée) Müll. Arg.
leuconephala (Nyl.) Zahlbr.

Graphis Adans.

afzelii Ach.
beaumontii Tuck.
elegans (Borr. *ex* Sm.) Ach.
pavoniana Fée
poitaeoides Nyl.
scripta (L.) Ach.
tenella Ach.

Gyrostomum Fr.

scyphuliferum (Ach.) Nyl.

Haematomma Mass.

puniceum (Sm. *ex* Ach.) Mass.
subpuniceum (Müll. Arg.) B. de Lesd.

Heppia Naeg.

lutosa (Ach.) Nyl.

Heterodermia Trev.

albicans (Pers.) Swinsc. & Krog
appalachensis (Kurok.) W. Culb.
diademata (Tayl.) Awas.
echinata (Tayl.) W. Culb.
galactophylla (Tuck.) W. Culb.
granulifera (Ach.) W. Culb.
hypoleuca (Mühl.) Trev.
leucomela (L.) Poelt
obscurata (Nyl.) Trev.
rugulosa (Kurok.) Wetm.
speciosa (Wulf.) Trev.

Hypotrachyna (Vain.) Hale

formosana (Zahlbr.) Hale
laevigata (Sm.) Hale
livida (Tayl.) Hale
pustulifera (Hale) Hale

Lasallia Mer.

papulosa (Ach.) Llano

Lecanora Ach.

allophana (Ach.) Röhl.
atra (Huds.) Ach.
caesiorubella Ach.
subsp. *glaucomodes* (Nyl.) Imsh. & Brodo
subsp. *lathamii* Imsh. & Brodo
subsp. *saximontana* Imsh. & Brodo
carpinea (L.) Vain
chrysoleuca (Sm.) Ach.
coilocarpa (Ach.) Nyl.
frustulosa (Dicks.) Ach.
glabrata (Ach.) Malme
melanophthalma (Ram.) Ram.
miculata Ach.
muralis (Schreb.) Rabenh.
peltata (Ram.) Steud.
subfusca (L.) Ach.
texana W. Web. *in ed.*
varia (Ehrh.) Ach.

Lecidea Ach.

coroniformis Kremp.
crenata (Tayl.) Stizenb.
decipiens (Hedw.) Ach.
globifera Ach.
hypomela Nyl.
icterica (Mont.) Tayl.
lapicida (Ach.) Ach.
lithophila (Ach.) Ach.
parvifolia Pers.
rufonigra (Tuck.) Nyl.
russellii Tuck.
russula Ach.
scalaris (Ach.) Ach.
[*Psora texana* W. Web.]
varians Ach.

Lepraria Ach.

chlorina (Ach.) Ach. *ex.* Sm.
membranacea (Dicks.) Vain.

Leptogium S. Gray

arsenei Sierk
austroamericanum (Malme) Dodge
azureum (Sw.) Mont.
chloromelum (Sw. *ex* Ach.) Nyl.
corticola (Tayl.) Tuck.
cyanescens (Ach.) Körb.
denticulatum Nyl.
furfuraceum (Harm.) Sierk
hirsutum Sierk
inflexum Nyl.
juniperinum Tuck.
lichenoides (L.) Zahlbr.

marginellum (Sw.) S. Gray
milligranum Sierk
phyllocarpum (Pers.) Mont.
platynum (Tuck.) Herre
plicatile (Ach.) Leight.
saturninum (Dicks.) Nyl.

Leptotrema Mont. & Bosch.

heterosporum (Knight) Zahlbr.
monosporum (Nyl.) Müll. Arg.
polycarpum Müll. Arg.
ravenelii (Tuck.) Fink
santense (Tuck.) Zahlbr.
wightii (Tayl.) Müll. Arg.

Lobaria Schreb.

quercizans Michx.
ravenelii (Tuck.) Yoshm.

Lopadium Körb.

augustini (Tuck.) Zahlbr.
domingense (Pers.) Fink
leucoxanthum (Spreng.) Zahlbr.

Medusulina Müll. Arg.

texana Müll. Arg.

Melanotheca Fée

aggregata (Fée) Müll. Arg.
cruenta (Mont.) Müll. Arg.

Melaspilea Nyl.

maculosa (Fr.) Müll. Arg.

Microthelia Körb.

micula Körb.

Mycocalicium Vain.

albonigrum (Nyl.) Fink

Nephroma Ach.

helveticum Ach.

Normandina Nyl.

pulchella (Borr.) Nyl.

Ocellularia Mey.

americana Hale
domingensis (Fée) Müll. Arg.
subtilis (Tuck.) Ridd.
stictidea (Nyl.) Vězda

Ochrolechia Mass.

pallescens (L.) Mass.
parella (L.) Mass.
tartarea (L.) Mass.

Opegrapha Ach.

astraea Tuck.
bonplandii Fée
prosodea Ach.

Pannaria Del.

lurida (Mont.) Nyl.
microphylla (Sw.) Mass.
pityrea (DC.) Degel.
rubiginosa (Thunb. *ex* Ach.) Del.

Parmelia Ach.

bolliana Müll. Arg.
chiricahuensis R. Anderson & W. Web.
exasperata De Not.
exasperatula Nyl.
flaventior Stirt.
hababiana Gyeln.
hypoleucites Nyl.
olivacea (L.) Ach.
perreticulata (Räs.) Hale
praesignis Nyl.
rudecta Ach.
subolivacea Nyl.
subpraesignis Nyl.
subrudecta Nyl.
ulophyllodes (Vain.) Sav.

Parmeliella Müll. Arg.

pannosa (Sw.) Nyl.

Parmelina Hale

aurulenta (Tuck.) Hale
dissecta (Nyl.) Hale
galbina (Ach.) Hale
horrescens (Tayl.) Hale
obsessa (Ach.) Hale

Parmeliopsis (Stizenb.) Nyl.

aleurites (Ach.) Nyl.
subambigua Gyeln.

Parmentaria Fée

astroidea Fee
ravenelii (Tuck.) Müll. Arg.

Parmotrema Mass.

austrosinense (Zahlbr.) Hale
cetratum (Ach.) Hale
conferendum Hale
crinitum (Ach.) Choisy
cristiferum (Tayl.) Hale
endosulphureum (Hillm.) Hale
eurysacum (Hue) Hale
haitiense (Hale) Hale
hypotropum (Nyl.) Hale

louisianae (Hale) Hale
margaritatum (Hue) Hale
mellissii (Dodge) Hale
michauxianum (Zahlbr.) Hale
perforatum (Jacq.) Mass.
praesorediosum (Nyl.) Hale
preperforatum (W. Culb.) Hale
rampoddense (Nyl.) Hale
reticulatum (Tayl.) Choisy
rigidum (Lynge) Hale
robustum (Degel.) Hale
subisidiosum (Müll. Arg.) Hale
subtinctorium (Zahlbr.) Hale
sulphuratum (Nees & Flot.) Hale
tinctorum (Nyl.) Hale
ultralucens (Krog) Hale

Peltigera Willd.

canina (L.) Willd.
collina (Ach.) Ach.
polydactyla (Neck.) Hoffm.
rufescens (Weiss) Humb.
spuria (Ach.) DC.

Peltula Nyl.

bolanderi (Tuck.) Wetm.
euploca (Ach.) Oz. & Clauz.
michoacanensis (B. de Lesd.) Wetm.
obscurans (Nyl.) Gyeln.
omphaliza (Nyl. *in* Eckf.) Wetm.
placodizans (Zahlbr.) Wetm.
polyspora (Tuck.) Wetm.
radicata (Ach.) Nyl.
richardsii (Herre) Wetm.

Pertusaria DC.

alpina Hepp
amara (Ach.) Nyl.
hymenea (Ach.) Schaer.
leioplaca (Ach.) DC.
multipuncta (Turn.) Nyl.
pertusa (L.) Tuck.
pustulata (Ach.) Duby
texana Müll. Arg.
tuberculifera Nyl.
velata (Turn.) Nyl.
xanthodes Müll. Arg.

Phaeographis Müll. Arg.

dendritica (Ach.) Müll. Arg.
exaltata (Mont.) Müll. Arg.

Phlyctis (Wallr.) Flot.

willeyi Tuck.

Physcia (Schreb.) DC.

aipolia (Ehrh.) Hampe
alba (Fée) Müll. Arg.
albicans (Pers.) Thoms.
albinea (Ach.) Nyl.
biziana (Mass.) Zahlbr.
callosa Nyl.
ciliata (Hoffm.) Du Rietz
clementi (Sm.) Maas G.
convexa Müll. Arg.
dubia (Hoffm.) Lett.
halei Thoms.
hirsuta Meresch.
hispidula (Ach.) Frey
intermedia Vain.
lacinulata Müll. Arg.
leana (Tuck.) Tuck.
mexicana B. de Lesd.
millegrana Degel.
nigricans (Flörke) Stizenb.
orbicularis (Neck.) Poetsch
rubropulchra (Degel.) Moberg
stellaris (L.) Nyl.
subtilis Degel.
tribacoides Nyl.
wainioi Räs.

Physciopsis Choisy

elaeina (Sm.) Poelt
syncolla (Tuck. *ex* Nyl.) Poelt

Physconia Poelt

grisea (Lam.) Poelt
pulverulenta (Schreb.) Poelt

Polyblastiopsis Zahlbr.

dispora (Müll. Arg.) Zahlbr.
lactea (Mass.) Zahlbr.

Porina Müll. Arg.

nucula Ach.
subcinerea (Nyl.) Zahlbr.

Pseudevernia Zopf

intensa (Nyl.) Hale & W. Culb

Pseudocyphellaria Vain

aurata (Ach.) Vain.

Pseudoparmelia Lynge

baltimorensis (Gyeln.) Hale
caperata (L.) Hale
caroliniana (Nyl.) Hale
cryptochlorophaea (Hale) Hale
rutidota (Hook. f. & Tayl.) Hale
sphaerospora (Nyl.) Hale
texana (Tuck.) Hale

Pyrenopsis Nyl.

viridirufa (Tuck.) Tuck.

Pyrenula Ach.

aspistea (Afz.) Ach.
mammillina (Ach.) Trev.
nitida (Weig.) Ach.

Pyxine Fr.

caesiopruinosa (Nyl.) Imsh.
eschweileri (Tuck.) Vain.
sorediata (Ach.) Mont.

Ramalina Ach.

complanata (Sw.) Ach.
denticulata (Eschw.) Nyl.
ecklonii (Spreng.) Mey. & Flot.
farinacea (L.) Ach.
fastigiata (Pers.) Ach.
fraxinea (L.) Ach.
montagnei De Not.
peruviana Ach.
pollinaria (Westr.) Ach.
sinensis Jatta
sorediantha Nyl.
stenospora Müll. Arg.
tenuis (Tuck.) Merr.
usnea (L.) Howe
willeyi Howe

Rechingeria Serv.

cribellifera (Nyl.) Serv.

Rhizocarpon Ram.

badioatrum (Flörke *ex* Spreng.) Th. Fr.

Rinodina Ram.

applanata Magn.
bischoffii (Hepp) Mass.
exigua (Ach.) S. Gray

Roccella DC.

babingtonii Mont.

Sarcographa Fée

tricosa (Ach.) Müll. Arg.

Sarcogyne Flot.

bolleana Magn.
clavus (Ram.) Kremp.
regularis Körb.

Schismatomma Mass.

ravenelii (Tuck.) Zahlbr.

Speerschneidera Trev.

euploca (Tuck.) Trev.

Sticta Schreb.

weigelii (Isert *ex* Ach.) Vain.

Strigula Fr.

complanata (Fée) Mont.
elegans (Fée) Müll. Arg.

Synalissa Fr.

symphorea (Ach.) Nyl.
texana Tuck.

Teloschistes Norm.

chrysophthalmus (L.) Th. Fr.
exilis (Michx.) Vain.
flavicans (Sw.) Norm.

Thelotrema Ach.

interpositum (Nyl.) Müll. Arg.
lepadium (Ach.) Ach.
sanfordianum Zahlbr.

Thyrea Mass.

nigritella Lett.
pulvinata (Schaer.) Mass.
pyrenoides (Nyl.) Fink

Trypethelium Spreng.

mastoideum Ach.
pallescens Fée
tropicum, (Ach.) Müll. Arg.
virens Tuck.

Umbilicaria Hoffm.

vellea (L.) Ach.

Usnea P. Br. & Adans.

angulata Ach.
antillarum (Vain.) Zahlbr.
arizonica Mot.
cavernosa Tuck.
cirrosa Mot.
dasypoga (Ach.) Röhl.
diplotypus Vain.
florida (L.) Wigg.
herrei Hale
hirta (L.) Wigg.
mutabilis Stirt.
rubiginea (Michx.) Mass.
strigosa (Ach.) A. Eat.
subfloridana Stirt.
trichodea Ach.

Verrucaria Schrad.

calciseda DC.
muralis Ach.

Xanthoparmelia (Vain.) Hale

conspersa (Ach.) Hale
cumberlandia (Gyeln.) Hale
dierythra (Hale) Hale

hypomelaena (Hale) Hale
kurokawae (Hale) Hale
lecanorica (Hale) Hale
lineola (Berry) Hale
mexicana (Gyeln.) Hale
novomexicana (Gyeln.) Hale
plittii (Gyeln.) Hale
psoromifera (Kurok.) Hale
subramigera (Gyeln.) Hale
taractica (Kremp.) Hale
tasmanica (Hook. f. & Tayl.) Hale
tinctina (Mah. & Gill.) Hale
tucsonensis (Nash) Egan
weberi (Hale) Hale

Xanthoria (Fr.) Th. Fr.

candelaria (L.) Th. Fr.
elegans (Link) Th. Fr.
fallax (Hepp) Arn.
parietina (L.) Th. Fr.
polycarpa (Ehrh.) Oliv.
ramulosa (Tuck.) Herre

SYNONYMS AND EXCLUDED NAMES

Acarospora flava (Bell.) Trev. = *Acarospora chlorophana* (Wahlehb. *ex* Ach.) Mass.
Acarospora squamulosa (Schrad.) Trev. = *Acarospora macrospora* (Hepp) Bagl.
Agyrium rufum (Pers.) Fr. = a fungus.
Anaptychia ciliaris (L.) Körb. Texas specimens actually *Speerschneidera euploca* (Tuck.) Trev.
Anaptychia domingensis (Ach.) Mass. = *Heterodermia albicans* (Pers.) Swinsc. & Krog
Anaptychia echinata (Tayl.) Kurok. = *Heterodermia echinata* (Tayl.) W. Culb.
Anaptychia granulifera (Ach.) Mass. = *Heterodermia granulifera* (Ach.) W. Culb.
Anaptychia hypoleuca (Mühl.) Mass. = *Heterodermia hypoleuca* (Mühl.) Trev.
Anaptychia leucomelaena (L.) Mass. = *Heterodermia leucomela* (L.) Poelt
Anaptychia obscurata (Nyl.) Vain. = *Heterodermia obscurata* (Nyl.) Trev.
Anaptychia "palmatula" auct. = *Anaptychia palmulata* (Michx.) Vain.
Anaptychia speciosa (Wulf.) Mass. = *Heterodermia speciosa* (Wulf.) Trev.
Anaptychia wrightii (Tuck.) Zahlbr. A *Heterodermia* species; synonymy uncertain.
Arthonia astrica Tuck. *ex* Will. Synonymy uncertain.
Arthonia interveniens Nyl. = *Arthothelium interveniens* (Nyl.) Zahlbr.
Arthonia macrotheca Fée = *Arthothelium macrothecum* (Fée) Mass.
Arthonia oxytera Nyl. Synonymy uncertain.
Bacidia icterica (Mont.) Tuck. = *Lecidea icterica* (Mont.) Tayl.
Biatora crenata (Tayl.) Tuck. = *Lecidea crenata* (Tayl.) Stizenb.
Biatora hypomela (Nyl.) Tuck. = *Lecidea hypomela* Nyl.
Biatora icterica Mont. = *Lecidea icterica* (Mont.) Tayl.
Biatora medialis Tuck. = *Bacidia medialis* (Tuck.) Zahlbr.
Biatora parvifolia (Pers.) Mont. = *Lecidea parvifolia* Pers.
[*Biatora rubella* var. *incompta*] = *Bacidia incompta* (Borr. *ex* Hook.) Anzi
Biatora rufonigra Tuck. = *Lecidea rufonigra* (Tuck.) Nyl.
Biatora russellii (Tuck.) Tuck. = *Lecidea russellii* Tuck.
Biatora schweinitzii (Tuck.) Tuck. = *Bacidia schweinitzii* (Tuck.) Schneid.
Biatora stigmatella Tuck. = *Bacidia stigmatella* (Tuck.) Zahlbr.
Biatora suffusa Fr. = *Bacidia suffusa* (Fr.) Schneid.
Biatora varians (Ach.) Eschw. = *Lecidea varians* Ach.
Biatorella nannaria (Tuck.) Zahlbr. Synonymy uncertain.
Blastenia diphasia (Tuck.) Zahlbr. = [*Caloplaca diphasia*]
Blastenia floridana (Tuck.) Zahlbr. = [*Caloplaca floridana*]
Blastenia pollinii Mass. = *Caloplaca pollinii* (Mass.) Jatta

Borrera chrysophthalma (L.) Ach. = *Teloschistes chrysophthalmus* (L.) Th. Fr.

Borrera exilis (Michx.) Ach. = *Teloschistes exilis* (Michx.) Vain.

Buellia badioatra (Flörke *ex* Spreng.) Mudd = *Rhizocarpon badioatrum* (Flörke *ex* Spreng.) Th. Fr.

Buellia inquilina Tuck. = a fungus.

Buellia parasema De Not. = *Buellia disciformis* (Fr.) Mudd.

Buellia parasema var. *triphragmia* (Nyl.) Th. Fr. = *Buellia lauri-cassiae* (Fée) Müll. Arg.

Buellia parasema var. *caesiopruinosa* = *Buellia disciformis* f. *caesiopruinosa* (Nyl.) Vain.

Buellia rinodinoides Anzi = misidentifications.

Buellia rinodinospora Riddle = *Buellia callispora* (Knight.) J. Stein.

Buelliella inquilina (Tuck.) Fink = a fungus.

Calicium albonigrum Nyl. = *Mycocalicium albonigrum* (Nyl.) Fink

Callopisma haematites (Chaub.) Mass. = *Caloplaca cerina* (Ehrh.) Th. Fr.

Caloplaca aurantiaca (Lightf.) Th. Fr. = *Caloplaca flavorubescens* (Huds.) Laundon

Caloplaca subnitida (Malme) Zahlbr. Synonymy uncertain.

[*Candelaria concolor* var. *stellata*] Synonymy uncertain but probably refers to *Candelaria concolor* (Dicks.) B. Stein.

Candelariella cerinella (Vain.) Zahlbr. = *Candelariella aurella* (Hoffm.) Zahlbr.

Cetraria fendleri f. *coralligera* W. Web. = *Cetraria coralligera* (W. Web.) Hale

Cladonia evansii Abb. = *Cladina evansii* (Abb.) Hale & W. Culb.

Cladonia impexa Harm. = *Cladina impexa* (Harm.) B. de Lesd. Texas records probably misidentifications.

Cladonia mitrula Tuck. = *Cladonia capitata* (Michx.) Spreng.

Cladonia pulchella Schwein. = *Cladonia didyma* (Fée) Vain.

Cladonia santensis var. *beaumontii* Tuck. = *Cladonia beaumontii* (Tuck.) Vain.

Cladonia subtenuis (Abb.) Evans = *Cladina subtenuis* (Abb.) Hale & W. Culb.

Coccocarpia micheneri Tuck. = *Coccocarpia erythroxili* (Spreng.) Swinsc. & Krog

Coccocarpia parmelioides (Hook.) Tuck. *ex* Curt. = *Coccocarpia erythroxili* (Spreng.) Swinsc. & Krog

Collema aggregata (Ach.) Röhl. = *Collema fasciculare* (L.) Wigg.

Collema nigrescens var. *leucopepla* Tuck. = *Collema leucopeplum* (Tuck.) Schneid.

Collema plicatile (Ach.) Ach. = *Leptogium plicatile* (Ach.) Lightf.

[*Collema pulchellum* var. *leucopeplum*] = *Leptogium corticola* (Tayl.) Tuck.

Crocynia lanuginosa (Ach.) Hue = *Lepraria membranacea* (Dicks.) Vain.

Dermatocarpon arboreum (Schwein.) Fink = *Dermatocarpon tuckermanii* (Rav.) Zahlbr.

Dirinaria aegialita (Ach.) B. Moore = *Dirinaria confusa* Awas. or *D. confluens* (Fr.) Awas. for Texas records.

Endocarpon moulinsii Mont. = *Dermatocarpon moulinsii* (Mont.) Zahlbr.

Endocarpon rufescens Ach. = *Dermatocarpon rufescens* (Ach.) Th. Fr.

Evernia furfuracea (L.) Mann = *Pseudevernia intensa* (Nyl.) Hale & W. Culb. for Texas records.

[*Gasparrinia eugyra*] = *Caloplaca eugyra* (Tuck.) Zahlbr.

Gasparrinia cirrochroa (Ach.) Stein = *Caloplaca cirrochroa* (Ach.) Th. Fr.

Graphis acharii Fée = *Graphina acharii* (Fée) Müll. Arg.

Graphis dendritica (Ach.) Ach. = *Phaeographis dendritica* (Ach.) Müll. Arg.

Graphis diversa Nyl. = *Phaeographis exaltata* (Mont.) Müll. Arg.

Graphis radiata (Mont.) Nyl. Described from French Guyana and Texas report questionable.

Graphis rigida f. *enteroleuca* Nyl. = *Graphina acharii* f. *enteroleuca* (Nyl.) Zahlbr.

Graphis scripta var. *serpentina* (L.) Mey. Synonymy uncertain.

Graphis scripta var. *sophistica* (Nyl.) Tuck. Synonymy uncertain.
Graphis scripta var. *tenella* (Ach.) Tuck. = *Graphis tennella* Ach.
Graphis tricosa Ach. = *Sarcographa tricosa* (Ach.) Müll. Arg.
Heppia despreauxii (Mont.) Tuck. = *Heppia lutosa* (Ach.) Nyl.
Heppia planescens Nyl. Synonymy uncertain.
Heterodermia domingensis (Ach.) Trev. = *Heterodermia albicans* (Pers.) Swinsc. & Krog
Heterodermia tremulans (Müll. Arg.) W. Culb. = *Heterodermia speciosa* (Wulf.) Trev.
Heterothecium domingense (Pers.) Tuck. = *Lopadium domingense* (Pers.) Fink
Heterothecium leucoxanthum (Spreng.) Mass. = *Lopadium leuxoxanthum* (Spreng.) Zahlbr.
Heterothecium nannarium Tuck. = *Biatorella nannaria* (Tuck.) Zahlbr. Synonymy uncertain.
Heterothecium pachycheilum (Tuck.) Tuck. = *Bombyliospora pachycheila* (Tuck.) Zahlbr.
Lasallia pustulata (L.) Mer. = *Lasallia papulosa* (Ach.) Llano for Texas specimens.
Lecanora aurantiaca (Lightf.) Fw. = *Caloplaca flavorubescens* (Huds.) Laundon
Lecanora calcarea (L.) Somm. = *Aspicilia calcarea* (L.) Mudd
Lecanora calcarea var. *contorta* (Hoffm.) Hepp = *Aspicilia contorta* (Hoffm.) Kremp.
Lecanora camptidia Tuck. = *Caloplaca camptidia* (Tuck.) Zahlbr.
Lecanora chlorophana (Wahlenb. *ex* Ach.) Ach. = *Acarospora chlorophana* (Wahlenb. *ex* Ach.) Mass.
Lecanora chonion Tuck. = *Lecidea crenata* (Tayl.) Stizenb.
Lecanora chrysops Tuck. = *Acarospora chrysops* (Tuck.) Magn.
Lecanora cinerea (L.) Somm. = *Aspicilia cinerea* (L.) Körb.
Lecanora diphasia Tuck. = [*Caloplaca diphasia*]
Lecanora floridana Tuck. = [*Caloplaca floridana*]
Lecanora pallescens (L.) Röhl. = *Ochrolechia pallescens* (L.) Mass.
Lecanora pallescens f. *variolosa* Fw. = *Ochrolechia pallescens* f. *variolosa* (Fw.) Jatta
Lecanora pallida var. *angulosa* (Schreb.) Rabenh. = *Lecanora carpinea* (L.) Vain.
Lecanora privigna var. *pruinosa* (Ach.) Tuck. = *Sarcogyne regularis* Körb.
Lecanora punicea (Sw. *ex* Ach.) Ach. = *Haematomma puniceum* (Sm. *ex* Ach.) Mass.
Lecanora rubina (Vill.) Ach. = *Lecanora chrysoleuca* (Sm.) Ach.
Lecanora rubina (Vill.) Ach. var. *heteromorpha* Ach. = *Lecanora peltata* (Ram.) Steud.
Lecanora rubina var. *opaca* (Ach.) Rabenh. = *Lecanora melanophthalma* (Ram.) Ram.
Lecanora subfusca var. *glabrata* Ach. = *Lecanora glabrata* (Ach.) Malme
Lecanora subfusca var. *vulgaris* Rabenh. = *Lecanora subfusca* (L.) Ach.
Lecanora wrightii Tuck. = *Lecidea icterica* (Mont.) Tayl.
Lecanora xanthophana Nyl. = *Acarospora xanthophana* (Nyl.) Jatta
Lecanora xanthophana f. *dealbata* Tuck. = *Acarospora xanthophana* (Nyl.) Jatta
Lecidea crenata f. *dealbata* (Tuck.) Zahlbr. = a recognized form.
Lecidea crenata var. *coroniformis* (Kremp.) Zahlbr. = *Lecidea coroniformis* Kremp.
Lecidea exigua Chaub. = (?) *Lecidea varians* Ach.
Lecidea pruinosa Ach. = *Lecidea lithophylla* (Ach.) Ach.
Leptogium bullatum (Sw. *ex* Ach.) Mont. Texas records probably misidentifications.
Leptogium palmatum (Huds.) Mont. Texas records probably misidentifications.
Leptogium pulchellum (Ach.) Nyl. = *Leptogium corticola* (Tayl.) Tuck.
Leptogium tremelloides (L.) S. Gray. Texas records misidentifications.
Lobaria erosa (Eschw.) Nyl. = *Lobaria ravenelii* (Tuck.) Yoshm.
[*Melaspilea angulosa*]. Synonymy uncertain.
Omphalaria pyrenoides Nyl. = *Thyrea pyrenoides* (Nyl.) Fink

Omphalaria texana (Tuck.) Tuck. = *Synalissa texana* Tuck.
Opegrapha tribulodes Tuck. = a fungus.
Pannaria molybdaea (Pers.) Tuck. = *Coccocarpia erythroxili* (Spreng.) Swinsc. & Krog
[*Pannaria molybdaea* var. *cronia*] = *Coccocarpia cronia* (Tuck.) Vain.
Parmelia andreana Müll. Arg. = *Parmelia flaventior* Stirt.
Parmelia aurulenta Tuck. = *Parmelina aurulenta* (Tuck.) Hale
Parmelia austrosinensis Zahlbr. = *Parmotrema austrosinense* (Zahlbr.) Hale
Parmelia borreri (Turn. *ex* Sm. & Sowerby) Turn. Texas records are misidentifications.
Parmelia borreri var. *hypomela* Tuck. = *Parmelia subpraesignis* Nyl.
Parmelia caperata (L.) Ach. = *Pseudoparmelia caperata* (L.) Hale
Parmelia caperata var. *incorrupta* (Moore) Berry = *Parmelia praesignis* Nyl.
Parmelia caperata var. *subglauca* (Gasilien) Nyl. = *Pseudoparmelia caperata* (L.) Hale
Parmelia caroliniana Nyl. = *Pseudoparmelia caroliniana* (Nyl.) Hale
Parmelia cetrata Ach. = *Parmotrema cetratum* (Ach.) Hale
Parmelia congruens Ach. = *Pseudoparmelia sphaerospora* (Nyl.) Hale
Parmelia conspersa (Ach.) Ach. = *Xanthoparmelia conspersa* (Ach.) Hale
Parmelia conspersa Chem. Strain 2 = *Xanthoparmelia lineola* (Berry) Hale
Parmelia conspersa Chem. Strain 3 = *Xanthoparmelia novomexicana* (Gyeln.) Hale
Parmelia conspersa var. *isidiata* (Anzi) Lindau = a *Xanthoparmelia* species.
Parmelia coronata Fée = *Bulbothrix coronata* (Fée) Hale
Parmelia crinita Ach. = *Parmotrema crinitum* (Ach.) Choisy
Parmelia cristifera Tayl. = *Parmotrema cristiferum* (Tayl.) Hale
Parmelia confoederata W. Culb. = *Bulbothrix confoederata* (W. Culb.) Hale
Parmelia coralloidea Vain. = *Parmotrema tinctorum* (Nyl.) Hale
Parmelia cubensis Nyl. = *Pseudoparmelia sphaerospora* (Nyl.) Hale
Parmelia cumberlandia (Gyeln.) Hale = *Xanthoparmelia cumberlandia* (Gyeln.) Hale
Parmelia dierythra Hale = *Xanthoparmelia dierythra* (Hale) Hale
Parmelia dissecta Nyl. = *Parmelina dissecta* (Nyl.) Hale
Parmelia endosulphurea (Hillm.) Hale = *Parmotrema endosulphureum* (Hillm.) Hale
Parmelia epiclada Hale = *Parmotrema michauxianum* (Zahlbr.) Hale
Parmelia erecta Berry = *Parmotrema perforatum* (Jacq.) Mass.
Parmelia eurysaca Hue = *Parmotrema eurysacum* (Hue) Hale
Parmelia finkii Zahlbr. = *Parmelina obsessa* (Ach.) Hale
Parmelia frondifera Merr. = *Parmelia bolliana* Müll. Arg.
Parmelia formosana Zahlbr. = *Hypotrachyna formosana* (Zahlbr.) Hale
Parmelia furfuracea (L.) Ach. = *Pseudevernia intensa* (Nyl.) Hale & W. Culb. for Texas records.
Parmelia galbina Ach. = *Parmelina galbina* (Ach.) Hale
Parmelia horrescens Tayl. = *Parmelina horrescens* (Tayl.) Hale
Parmelia hubrichtii Berry = *Parmelina dissecta* (Nyl.) Hale
Parmelia hypotropa Nyl. = *Parmotrema hypotropum* (Nyl.) Hale
Parmelia hypotropa var. *sorediata* Müll. Arg. = *Parmotrema hypotropum* (Nyl.) Hale
Parmelia hypotropoides Nyl. *ex* Will. = *Parmotrema perforatum* (Jacq.) Mass.
Parmelia isidiata (Anzi) Gyeln. Chem. Strain 2 = *Xanthoparmelia mexicana* (Gyeln.) Hale
Parmelia kurokawae Hale = *Xanthoparmelia kurokawae* (Hale) Hale
Parmelia laevigata (Sm.) Ach. = *Hypotrachyna laevigata* (Sm.) Hale
Parmelia latissima Fée. Misidentifications for Texas.
Parmelia lecanorica Hale = *Xanthoparmelia lecanorica* (Hale) Hale

Parmelia leucochlora Tuck. *ex* Nyl. = *Pseudoparmelia sphaerospora* (Nyl.) Hale

Parmelia lineola Berry = *Xanthoparmelia lineola* (Berry) Hale

Parmelia livida Tayl. = *Hypotrachyna livida* (Tayl.) Hale

Parmelia margaritata Hue = *Parmotrema margaritatum* (Hue) Hale

Parmelia mellissii Dodge = *Parmotrema mellissii* (Dodge) Hale

Parmelia mesogens Nyl. = *Parmotrema mesogens* (Nyl.) Hale. Texas report probably a misidentification. Known only from Mexico.

Parmelia mexicana Gyeln. = *Xanthoparmelia mexicana* (Gyeln.) Hale

Parmelia michauxiana Zahlbr. = *Parmotrema michauxianum* (Zahlbr.) Hale

Parmelia novomexicana Gyeln. = *Xanthoparmelia novomexicana* (Gyeln.) Hale

Parmelia obsessa Ach. = *Parmelina obsessa* (Ach.) Hale

Parmelia olivacea var. *aspidota* Ach. = *Parmelia exasperata* De Not.

Parmelia perforata (Jacq.) Ach. = *Parmotrema perforatum* (Jacq.) Mass.

[*Parmelia perforata* var. *hypotropa*] = *Parmotrema hypotropum* (Nyl.) Hale

Parmelia perlata (Huds.) Ach. = *Parmotrema perlatum* (Huds.) Choisy

Parmelia perlata var. *ciliata* (Lam. *ex* Lam. & DC.) Duby. Synonymy uncertain.

Parmelia praesorediosa Nyl. = *Parmotrema praesorediosum* (Nyl.) Hale

Parmelia preperforata W. Culb. = *Parmotrema preperforatum* (W. Culb.) Hale

Parmelia proboscidea Tayl. = *Parmotrema crinitum* (Ach.) Choisy

Parmelia psoromifera Kurok. = *Xanthoparmelia psoromifera* (Kurok.) Hale

Parmelia pustulifera Hale = *Hypotrachyna pustulifera* (Hale) Hale

Parmelia quercina (Willd.) Vain. = *Parmelina quercina* (Willd.) Hale. Texas records probably misidentifications.

Parmelia quercina f. *minor* (Kremp.) Zahlbr. Synonymy uncertain.

Parmelia rampoddensis Nyl. = *Parmotrema rampoddense* (Nyl.) Hale

Parmelia reticulata Tayl. = *Parmotrema reticulatum* (Tayl.) Choisy

Parmelia rigida Lynge = *Parmotrema rigidum* (Lynge) Hale

Parmelia rutidota Hook. f. & Tayl. = *Pseudoparmelia rutidota* (Hook. f. & Tayl.) Hale

Parmelia scortella Nyl. = *Bulbothrix goebelii* (Zenker *in* Goebel & Kunze) Hale

[*Parmelia* (Subsection *Pyxine*) *sorediata*] = *Pyxine sorediata* (Ach.) Mont.

Parmelia soredica Nyl. = *Parmelia flaventior* Stirt.

Parmelia subcrinita Nyl. = *Parmotrema ultralucens* (Krog) Hale

Parmelia subisidiosa (Müll. Arg.) Dodge = *Parmotrema subisidiosum* (Müll. Arg.) Hale

Parmelia sublaevigata (Nyl.) Nyl. = *Hypotrachyna sublaevigata* (Nyl.) Hale. Texas records probably misidentifications of *H. livida* (Tayl.) Hale.

Parmelia subtinctoria Zahlbr. = *Parmotrema subtinctorium* (Zahlbr.) Hale

Parmelia sulphurata Nees & Flot. = *Parmotrema sulphuratum* (Nees & Flot.) Hale

Parmelia taractica Kremp. = *Xanthoparmelia taractica* (Kremp.) Hale

Parmelia tasmanica Hook. f. & Tayl. = *Xanthoparmelia tasmanica* (Hook. f. & Tayl.) Hale

Parmelia texana Tuck. = *Pseudoparmelia texana* (Tuck.) Hale

Parmelia tiliacea var. *minor* Müll. Arg. = *Parmelina galbina* (Ach.) Hale

Parmelia tinctoria Nyl. = missapplication of *P. tinctorum* = *Parmotrema tinctorum* (Nyl.) Hale

Parmelia tinctorum Nyl. = *Parmotrema tinctorum* (Nyl.) Hale

Parmelia ultralucens Krog = *Parmotrema ultralucens* (Krog) Hale

Parmeliopsis halei (Tuck.) Hale = *Parmeliopsis subambigua* Gyeln.

Peltigera canina var. *rufescens* (Weiss) Mudd = *Peltigera rufescens* (Weiss) Humb.

Peltigera canina var. *spuria* (Ach.) Schaer. = *Peltigera spuria* (Ach.) DC.

Pertusaria communis Lam. *ex* Lam. & DC. = *Pertusaria pertusa* (L.) Tuck.

Pertusaria leioplaca var. *octospora* Nyl. = *Pertusaria alpina* Hepp

Pertusaria melaleuca (Turn. & Borr.) Duby = *Pertusaria pustulata* (Ach.) Duby

[*Patellaria* (Sect. *Bacidia*) *rubella* var. *suffusa*] = *Bacidia suffusa* (Fr.) Schneid.

Physcia adglutinata (Flörke) Nyl. = *Physciopsis elaeina* (Sm.) Poelt

Physcia aegialita (Ach.) Nyl. = *Dirinaria confusa* Awas. for most Texas reports.

Physcia applanata (Fée) Zahlbr. = *Dirinaria applanata* (Fée) Awas.

Physcia astroidea (Clem.) Nyl. = *Physcia clementi* (Sm.) Maas G.

Physcia candelaria var. *stellata* Tuck. = *Candelaria stellata* (Tuck.) Müll. Arg. Probably refers to *Candelaria concolor* (Dicks.) B. Stein.

Physcia comosa (Eschw.) Nyl. = *Heterodermia galactophylla* (Tuck.) W. Culb. for Texas records.

Physcia chrysophthalma (L.) DC. = *Teloschistes chrysophthalmus* (L.) Th. Fr.

Physcia chrysophthalmus var. *pubera* (Ach.) Nyl. = *Teloschistes flavicans* var. *puberus* (Ach.) Müll. Arg.

Physcia crispa (Pers.) Nyl. = *Physcia albicans* (Pers.) Thoms.

Physcia elaeina (Sm.) A.L. Sm. = *Physciopsis elaeina* (Sm.) Poelt

Physcia endochrysea (Hampe) Nyl. = *Physcia rubropulchra* (Degel.) Moberg

Physcia euploca Tuck. = *Speerschneidera euploca* (Tuck.) Trev.

Physcia grisea (Lam.) Zahlbr. = *Physconia grisea* (Lam.) Poelt

Physcia hypoleuca (Mühl.) Tuck. = *Heterodermia hypoleuca* (Mühl.) Trev.

Physcia obscura (Ehrh.) Hampe = *Physcia ciliata* (Hoffm.) Du Rietz

Physcia obscura var. *adglutinata* (Flörke) Nyl. = *Physciopsis elaeina* (Sm.) Poelt

Physcia obscura var. *ciliata* (Hoffm.) Tuck. = *Physcia ciliata* (Hoffm.) Du Rietz

Physcia obscura f. *endochrysea* Nyl. = *Physcia rubropulchra* (Degel.) Moberg

Physcia obscura var. *sciastrella* (Nyl.) Oliv. = *Physcia nigricans* (Flörke) Stizenb.

Physcia orbicularis f. *albociliata* (B. de Lesd.) Thoms. = *Physcia hirsuta* Meresch.

Physcia parietina var. *laciniosa* (Duf. *ex* Schaer.) Mass. = *Xanthoria candelaria* var. *laciniosa* (Duf. *ex* Schaer.) Arn.

Physcia picta (Sw.) Nyl. = *Dirinaria picta* (Sw.) Clem. & Schear.

Physcia picta f. *erythrocardia* (Tuck.) Thoms. = *Dirinaria leopoldii* (Stein) Awas.

Physcia pulverulenta (Schreb.) Hampe = *Physconia pulverulenta* (Schreb.) Poelt

Physcia pulverulenta var. *angustata* (Hoffm.) Nyl. = *Physconia pulverulenta* (Schreb.) Poelt

Physcia pulverulenta var. *pityrea* Stizenb. = *Physconia grisea* (Lam.) Poelt

Physcia ravenelii Tuck. = *Heterodermia albicans* (Pers.) Swinsc. & Krog

Physcia setosa (Ach.) Nyl. = *Physcia hispidula* (Ach.) Frey

Physcia speciosa var. *granulifera* (Ach.) Tuck. = *Heterodermia granulifera* (Ach.) W. Culb.

Physcia speciosa var. *hypoleuca* (Mühl.) Nyl. = *Heterodermia hypoleuca* (Mühl.) Trev.

Physcia stellaris var. *angustata* Nyl. = *Physcia aipolia* f. *angustata* (Nyl.) Vain.

Physcia stellaris var. *astroidea* (Clem. *ex* Fr.) Tuck. = *Physcia clementi* (Sm.) Maas G.

Physcia stellaris var. *domingensis* (Ach.) Tuck. = *Heterodermia albicans* (Pers.) Swinsc. & Krog

Physcia syncolla Tuck. *ex* Nyl. = *Physciopsis syncolla* (Tuck. *ex* Nyl.) Poelt

[*Physcia wrightii*] = a *Heterodermia* species. Synonymy uncertain.

Placodium aurantiacum (Lightf.) Anzi = *Caloplaca flavorubescens* (Huds.) Laundon

Placodium camptidium (Tuck.) Tuck. = *Caloplaca camptidia* (Tuck.) Zahlbr.

Placodium cerinum (Hoffm.) Naeg. *ex* Hepp = *Caloplaca holocarpa* (Hoffm.) Wade

Placodium cinnabarinum (Ach.) Nyl. = *Caloplaca cinnabarina* (Ach.) Zahlbr.

Placodium diphasium (Tuck.) Tuck. = [*Caloplaca diphasia*]

Placodium elegans (Link) DC. *ex* Lam. & DC. = *Xanthoria elegans* (Link) Th. Fr.

Placodium eugyrum Tuck. = *Caloplaca eugyra* (Tuck.) Zahlbr.
Placodium ferrugineum (Huds.) Rabenh. = *Caloplaca ferruginea* (Huds.) Th. Fr.
Placodium ferrugineum var. *wrightii* Will. *ex* Tuck. = *Caloplaca wrightii* (Will. *ex.* Tuck.) Fink
Placodium floridanum (Tuck.) Tuck. = [*Caloplaca floridana*]
Placodium microphyllinum Tuck. = *Caloplaca microphyllina* (Tuck.) Hasse
Placodium vitellinum (Ehrh.) Hepp = *Candelariella vitellina* (Ehrh.) Müll. Arg.
Platygrapha ravenelii Tuck. = *Schismatomma ravenelii* (Tuck.) Zahlbr.
Polyblastia dispora Müll. Arg. = *Polyblastiopsis dispora* (Müll. Arg.) Zahlbr.
Pseudevernia consocians (Vain.) Hale & W. Culb. Texas records probably misidentifications.
Psora crenata (Tayl.) Reinke = *Lecidea crenata* (Tayl.) Stizenb.
Psora icterica (Mont.) Müll. Arg. = *Lecidea icterica* (Mont.) Tayl.
Psora texana W. Web. = [*Lecidea texana*]
[*Pyrenastrum astroideum*] = *Parmentaria astroidea* Fee
Pyrenastrum gemmeum Tuck. = *Parmentaria astroidea* Fée
Pyrenodesmia aurantiaca (Lightf.) Dodge & Baker = *Caloplaca flavorubescens* (Huds.) Laundon
[*Pyrenodesmia camptidia*] = *Caloplaca camptidia* (Tuck.) Zahlbr.
[*Pyrenodesmia cerina*] = *Caloplaca cerina* (Ehrh.) Th. Fr.
[*Pyrenodesmia cinnabarina*] = *Caloplaca cinnabarina* (Ach.) Zahlbr.
[*Pyrenodesmia diphasia*] = [*Caloplaca diphasia*]
[*Pyrenodesmia flavovirescens*] = *Caloplaca flavovirescens* (Wulf.) Dalla Torre & Sarnth.
[*Pyrenodesmia wrightii*] = *Caloplaca wrightii* (Will.) Fink
Pyrenula aggregata (Fée) Fée = *Melanotheca aggregata* (Fée) Müll. Arg.
Pyrenula cinchonae (Fée) Fée = *Pyrenula mamillina* (Ach.) Trev.
Pyrenula lactea (Mass.) Tuck. = *Polyblastiopsis lactea* (Mass.) Zahlbr.
Pyrenula mammillina var. *santensis* (Nyl.) Trev. = *Pyrenula mammillina* (Ach.) Trev.
Pyrenula nitida var. *nitidella* Flörke *ex* Schaer. = an accepted variety.
[*Pyrenula nitidella* var. *chlorospila*]. Synonymy uncertain.
Pyrenula pachycheila Tuck. = *Anthracothecium pyrenuloides* (Mont.) Müll. Arg.
Pyrenula subcinerea (Nyl.) Tuck. = *Porina subcinerea* (Nyl.) Zahlbr.
Pyxine cocoes var. *sorediata* (Ach.) Tuck. = *Pyxine sorediata* (Ach.) Mont.
Pyxine picta (Sw.) Tuck. = *Dirinaria picta* (Sw.) Clem. & Schear.
Pyxine picta var. *erythrocardia* Tuck. = *Dirinaria leopoldii* (Stein) Awas.
Ramalina calcicaris Mont. = *Ramalina montagnei* De Not.
Ramalina laevigata Fr. = *Ramalina ecklonii* (Spreng.) Mey. & Flot. for Texas Records.
Ramalina rigida (Pers.) Nyl. = *Ramalina willeyi* Howe
Ramalina rigida var. *montagnei* (De Not.) Tuck. = *Ramalina montagnei* De Not.
Ramalina yemensis (Ach.) Nyl. = *Ramalina ecklonii* (Spreng.) Mey. & Flot.
Ramalina yemensis var. *membranacea* (Laur.) Nyl. = *Ramalina ecklonii* var. *membranacea* (Laur.) Müll. Arg.
Rinodina novomexicana B. de Lesd. = *Dimelaena oreina* (Ach.) Norm.
Rinodina oreina (Ach.) Mass. = *Dimelaena oreina* (Ach.) Norm.
Rinodina suboreina B. de Lesd. = *Dimelaena oreina* (Ach.) Norm.
Sticta amplissima (Scop.) Rabenh. = *Lobaria ravenelii* (Tuck.) Yosh. or *Lobaria quercizans* Michx. for Texas records.
Sticta aurata Ach. = *Pseudocyphellaria aurata* (Ach.) Vain.
Sticta quercizans Ach. = *Lobaria quercizans* Michx.
[*Synalissa texana*]. Synonymy uncertain.
Synalissa viridirufa Tuck. = *Pyrenopsis viridirufa* (Tuck.) Tuck.

Synechoblastus coccophorus (Tuck.) Fink = *Collema coccophorum* Tuck.
Synechoblastus leptaleus (Tuck.) Fink = *Collema leptaleum* Tuck.
Synechoblastus texanus (Tuck.) Müll. Arg. = *Collema texanum* Tuck.
Teloschistes candelarius (L.) Fink = *Xanthoria candelaria* (L.) Th. Fr.
Teloschistes chrysophthalmus var. *flavicans* (Sw.) Tuck. = *Teloschistes flavicans* (Sw.) Norm.
Teloschistes concolor (Dicks.) Tuck. = *Candelaria concolor* (Dicks.) B. Stein
Teloschistes euplocus (Tuck.) Zahlbr. = *Speerschneidera euploca* (Tuck.) Trev.
Teloschistes lychneus f. *laciniosa* (Duf.) Tuck. = *Xanthoria candelaria* var. *laciniosa* (Duf.) Arn.
[*Thelotrema clavatum*]. Name apparently never validly published *fide* M.E. Hale.
Thelotrema monosporum Nyl. = *Leptotrema monosporum* (Nyl.) Müll. Arg.
Thelotrema subtile Tuck. = *Ocellularia subtilis* (Tuck.) Ridd.
Thelotrema texanum Will. *ex* Nyl. = *Ocellularia stictidea* (Nyl.) Vězda
Thelotrema wightii (Tayl.) Nyl. = *Leptotrema wightii* (Tayl.) Müll. Arg.
Trypethelium cruentum Mont. = *Melanotheca cruenta* (Mont.) Müll. Arg.
Umbilicaria pustulata (L.) Hoffm. = *Lasallia papulosa* (Ach.) Llano for Texas reports.
Usnea barbata var. *dasypoga* (Ach.) Ach. = *Usnea dasypoga* (Ach.) Röhl.
Usnea barbata var. *hirta* (L.) Fr. = *Usnea hirta* (L.) Wigg.
Usnea barbata var. *strigosa* (Ach.) Flot. = *Usnea strigosa* (Ach.) A. Eat.
Usnea trichodea var. *ciliata* Müll. Arg. Synonymy uncertain.
Verrucaria fusconigricans Nyl. *ex* Hue. Synonymy uncertain.
Verrucaria rupestris Schrad. = *Verrucaria muralis* Ach.
Xanthoria lynchnea (Ach.) Th. Fr. = *Xanthoria candelaria* (L.) Th. Fr.
Xanthoria lychnea var. *laciniata* (Duf.) Herre = *Xanthoria candelaria* var. *laciniosa* (Duf.) Arn.
[*Xanthoria parietina* var. *ramulosa*] = *Xanthoria ramulosa* Herre
Xanthoria substellaris (Ach.) Vain. = *Xanthoria fallax* (Hepp) Arn.

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SOME MICROALGAE FROM SAND[1]

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ABSTRACT

Forty-five samples of sand from the coast of Oahu (Hawaii) and the dunes (and immediate vicinity) of Sandhills State Park, Ward County, Texas, were assayed comparatively for the presence of algae by the technique of enrichment culture. Representatives of blue-green and green algae, and some diatoms were recognized and tabulated. *Schizothrix* was the most frequently encountered blue-green alga, while, as in previous investigations, *Chlamydomonas, Chlorococcum, Chlorosarcinopsis,* and *Tetracystis* were the most frequent green algae. Some of the organisms present in the dunes of Sandhills State Park are illustrated.

INTRODUCTION

That diatoms, dinoflagellates, blue-green and some red algae occur in coastal sands has frequently been reported (Round, 1965). However, reports of green algae other than *Ulothrix* in such a habitat are few (e.g., Archibald and Bold, 1975; Dykstra, *et al.,* 1975). Furthermore, Round (1965) states: "Sand beaches above high tide marks are usually so desiccated that algal species are rare." This may be true insofar as direct observation of actively growing algae is concerned, but the technique of enrichment culture contradicts Round's statement, as is clear from the results of the reports cited above, and the present one, in which surprisingly large numbers of algae are listed as occurring in desiccated (and moist) sand.

In an effort to augment our knowledge of the algal flora of sands, samples of the latter from 2 very different habitats were assayed for algae by the technique of enrichment culture. Samples were taken of both moist and desiccated (above high tide level) coastal sands from the island of Oahu, Hawaii. These were collected in June, 1976, and assayed for algae the same year. By contrast, samples

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of extremely desiccated sand were collected on June 24, 1977, from the dunes at Sandhills State Park near Monahans, Ward County, Texas (Eifler, 1970) and assayed for algae during the same summer. The Monahans sands are quarternary, "gray, unconsolidated, and largely lacking in clay and fine silt particles" (Huffington and Albritton, 1941). Samples were taken from both the bare dunes and, for comparison, from sand covered with vegetation (*Quercus havardii* and other small shrubs). A few samples were collected, for comparison, from dry, sandy roadside ditches near, but not in, Sandhills State Park. The Monahans sands are from an arid area (annual rainfall ca. 11.52 in), while the Oahu coastal sands were either moist (with sea water or rain) at the time of collection or from dry dune areas but still subject to frequent rains (annual rainfall, 25.28 in), sea-spray, dews and fogs.

MATERIALS AND METHODS

A total of 45 samples was collected and processed by the technique of enrichment culture. In this procedure aliquots of the samples were introduced into flasks containing either sterile Bold's Basal Medium (BBM) (Bischoff and Bold, 1963) or into an enriched sea water medium (von Stosch medium [VSE], [Stosch, 1964]). The flasks were then placed on the shelves of an illuminated (ca. 300 ft c, 12 hr lt, 12 hr dark) constant-temperature (20-22 C) culture chamber. When algae appeared in the flasks they were studied microscopically, while some were streaked on petri dishes of BBM agar and incubated in the light. From these plates, unialgal and/or axenic colonies were subsequently isolated and inoculated separately into tubes of sterile media for further study.

RESULTS

Table 1 summarizes the occurrence of blue-green and green algae in the 45 samples of sand from Oahu, Hawaii, and Sandhills State Park, Texas as they developed in enrichment culture. A number of conclusions may be inferred from the data in Table 1. First, representatives of genera of blue-green (5) and green algae (15) developed from the Hawaiian sand samples, compare favorably in number (7, 11) with those from Sandhills State Park, despite the greater annual rainfall (25.28 in) and less extreme temperatures (range, 52-90 F) on Hawaiian beaches as compared with those at Sandhills State Park (11.52 in rainfall, temperature range -10 F to 116 F). It is also obvious that fewer (1/4 - 1/2 as many) algae developed when the sand samples were flooded with a marine enrichment medium (VSE) as compared with one of low salinity (BBM).

The development of a species of the marine alga, *Enteromorpha*, in a BBM-enriched Hawaiian sample is not surprising, however, for the Oahu sand undoubtedly contained an abundance of inorganic salts and, furthermore, *Enteromorpha* is tolerant of a great range of salinities (Smith, 1950).

TABLE 1

Summary of the Blue-Green and Green Algae Developing from Sand Samples in Enrichment Culture (Figures Represent the Number of Enrichment Cultures in Which Representatives of each Genus Appeared)

	Oahu		Monahans Dunes					
			Exposed sand		Sand covered by vegetation		Roadside Ditch Near Dunes	
	BBM	VSE	BBM	VSE	BBM	VSE	BBM	VSE
Cyanochloronta								
Anabaena	4					1		
Aphanocapsa				3				
Lyngbya							1	
Nodularia	1		1					
Nostoc	3				2	3	1	
Oscillatoria	1				1	1	1	
Schizothrix	12		3	3	5		1	1
Chlorophycophyta								
Bracteacoccus			1					
Characium					3			
Chlamydomonas	8	1	4		3		1	
Chlorella	3							
Chlorococcum	7		6		4		2	
Chlorosarcinopsis	5	3	10		4		1	
Crucigenia	1							
Dunaliella		1						
Enteromorpha	1							
Gonium					1			
Klebsormidium	1							
Neochloris		1						
Protosiphon	1							
Pseudotetracystis							1	
Radiosphaera	1						1	
Rhizoclonium	1							
Spongiochloris	1		2					
Spongiococcum			1			3		
Stichococcus	1							
Tetracystis	8	1	4		1			1
Diatoms	5	1	1		3		1	
Totals (excluding diatoms)	18	5	9	2	9	4	9	2

Genera represented	Blue-Green Algae	Green Algae	Totals
Oahu Samples	5	15	20
Sand Dune Samples	7	11	18

Most of the algae which developed in the enrichment cultures were among those often present in soils, with 2 notable exceptions. The most surprising of these was the development of a species of the motile, colonial Volvocalean alga, *Gonium*, a typical aquatic, from a desiccated sand sample taken from the top of a sand dune in Sandhills State Park. The other was *Rhizoclonium*, another typically aquatic alga.

A total of 7 blue-green (Cyanochloronta) and 20 green (Chlorophycophyta) algae developed in the samples (in addition to a number of unidentified diatoms, fungi, bacteria and protozoa). Of the blue-green algae, the one which developed most frequently in both the Hawaiian and Sandhills samples was *Schizothrix calcicola*,[2] while, by contrast, a species of *Nodularia* appeared only once. It was of interest that *Schizothrix calcicola* adhered to and bound together the sand particles in the enrichment culture. *Nostoc* species developed only in those Sandhills samples which had been collected under vegetation but not in sand samples exposed to the sun. It is of interest that *Nostoc* developed when both BBM and VSE were the enriching media (Table 1).

Of the green algae, *Chlamydomonas, Chlorococcum, Chlorosarcinopsis* and *Tetracystis* appeared most frequently in the enrichment cultures, while a number (e.g., *Crucigenia, Klebsormidium,* etc.) were present only once. Although fewer genera of green algae were represented in the flora of the Sandhills State Park, as compared with those (15) in the Hawaiian sands, 2 (*Characium* and *Gonium*) occurred in the Texas samples and not in the Hawaiian.

One further trend, with reference to the blue-green algae, seems clear from the data in Table 1: More blue-green algae algae occur in soils covered by vegetation than in bare sand.

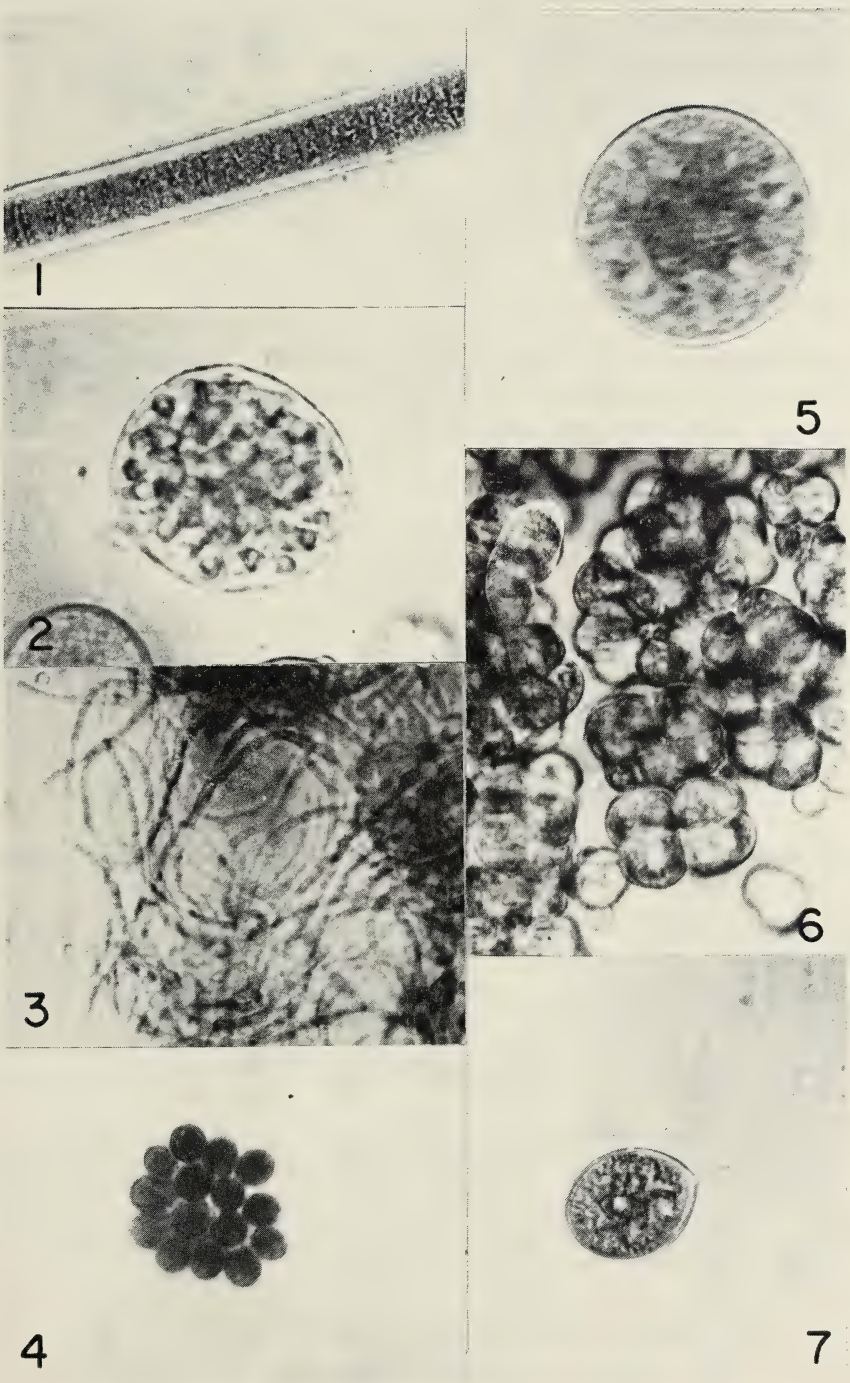
Although the development in a marine enrichment medium of *Dunaliella* from the Oahu coastal sands was to be expected, that of *Chlorosarcinopsis, Neochloris* and *Tetracystis* was surprising. Even more so, however, was the development in the marine medium of 6 different algae in the noncoastal sands of Sandhills State Park.

Because one usually does not consider sand as harboring many algae, photomicrographs of representatives of some of the genera encountered in the present investigation are included herewith as Figures 1-7.

DISCUSSION

Evidence for the occurrence of a variety of algae in sands, both inland and coastal, is increasing (Chantanachat and Bold, 1962; Archibald and Bold, 1975; Dykstra, *et al.,* 1975) and the present report augments this evidence, even though a lesser variety of algae has been encountered in the present investigation both in the Hawaiian and Sandhills State Park samples. This may be explained, in part,

[2]Kindly identified by Dr. Francis Drouet, Academy of Natural Sciences, Philadelphia, PA.



Figures 1–7.

Figs. 1–7. (continued)

Figure 1. *Lyngbya* sp., a blue-green alga; note priminent sheath, x 425.

Figure 2. *Nostoc* sp., a blue-green alga, young colony, x 425.

Figure 3. *Schizothrix calcicola* Gom., a filamentous blue-green alga, x 500.

Figure 4. *Gonium pectorale* Muell., a flagellate colonial green alga, x 600.

Figure 5. *Radiosphaera* sp., a nonmotile green alga, x 800.

Figure 6. *Chlorosarcinopsis* sp., a nonmotile green alga, x 1800.

Figure 7. *Characium* sp., an attached, unicellular green alga, x 1200.

by the smaller number of samples available to the authors of this paper, and/or in part by the long periods without rainfall to which the Sandhills State Park is subject. Through many years of study of edaphic algae, it has become clear to one of us (H.C.B.) that the frequency and diversity of edaphic algae are closely correlated with abundance of soil moisture. The same algae (*Chlamydamonas, Chlorococcum, Chlorosarcinopsis, Tetracystis* and *Schizothrix*) were once again those most frequently encountered in the Texas and Hawaiian sands. The presence of *Gonium*, an aquatic green alga, in a sample from exposed dunes, must be regarded as fortuitous, although in an earlier investigation (Dykstra, *et al.*, 1975), it was also reported once from a Texas coastal dune and once again (MacEntee, *et al.*, 1972) from the Edwards Plateau near Austin, Texas, from soil under *Juniperus ashei*.

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ENVIRONMENTAL FACTORS AFFECTING GROWTH AND DEVELOPMENT OF THE TEXAS MADRONE. I. EFFECT OF LIGHT INTENSITY ON SEEDLING GROWTH

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ABSTRACT

Seeds of the Texas madrone (*Arbutus xalapensis* H.B.K., var. *texana* Buckl.) were germinated in Fertl-Cubes and transferred to baskets when the cotyledons had begun to expand. The baskets were placed in a growth chamber and the seedlings were grown for 11 wks under the following conditions: 12-hr photoperiod, temperatures of 27 C during the day and 18 C at night, 60 to 70% relative humidity, and light intensity of 17,200, 12,900, or 8,600 lux. Plant height, fresh weight, dry weight and chlorophyll content was found to decrease with increasing light intensity, but % dry weight remained the same in all treatments. The data suggest that plant growth at the higher light intensities was reduced by the photobleaching of chlorophyll.

INTRODUCTION

The Texas madrone (*Arbutus xalapensis* H.B.K., var. *texana* Buckl.), considered to be an endangered species by the Texas Organization for Endangered Species, has attracted the attention of several workers, but research efforts have been hampered by the difficulty of getting the tree to survive past the seedling stage (Wiedenfeld, 1975). However, a technique that has been described recently (Hardesty and Whitenberg, 1976) allows the trees to be propagated successfully from seeds and permits long-term studies of the Texas madrone.

We have observed that, under natural conditions, madrone seedlings almost invariably grow under the protection of another tree. In the Edwards Plateau region, madrone seedlings are usually found near the base of a juniper or where juniper mulch is fairly thick. This observation suggests, among other possibilities,

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that light intensity is a critical factor in seedling survival, that the juniper mulch has sufficient water-holding capacity for establishment of the seedlings, or that a combination of light intensity and water relations is involved. This paper is concerned with the effect of light intensity on seedling growth.

METHODS AND MATERIALS

Berries were collected from madrones in the Sabinal Canyon area during November, 1976. The seeds were removed and germinated in moist Fertl-Cubes (Park Seed Co., Greenwood, S.C.), and the cubes were transferred to wooden baskets (approximately 16 cm x 13 cm high) filled with potting medium when the cotyledons of the seedlings had begun to expand. The potting medium was composed of peat moss:vermiculite:perlite:shredded juniper bark (2:1:1:1 v/v).

The baskets were placed in a growth chamber and the seedlings were grown under the following conditions: 12-hr photoperiod furnished by a mixture of cool white fluorescent tubes and incandescent bulbs; daytime temperature of 27 C and nighttime temperature of 18 C; 60% to 70% relative humidity and light intensities of 17,200 lux (HL), 12,900 lux (ML), or 8,600 lux (LL). Each light treatment was replicated 4 times. The plants were watered once every 2 weeks with 1/4 strength Hoagland's solution and at other times with deionized water. Plant heights were measured periodically during the growth period and after 11 weeks the plants were harvested and were fractionated into stems and leaves. Fresh and dry weights were obtained, and chlorophyll content of the fresh leaves was determined by the method of Brüinsma (1963). Meaningful root samples could not be obtained because the plant produces a very fine, fibrous root system that could not be separated from the potting medium.

RESULTS

Plant height, fresh weight, dry weight, and chlorophyll concentration decreased as light intensity increased (Table 1). As compared to the LL treatment, plants grown in HL were 67% as tall, produced 56% of the fresh and dry weight, and had 75% the chlorophyll concentration. Similarly, plants of the ML treatment were 82% as tall, produced 60% of the fresh weight and 62% of the dry weight, and had 84% of the chlorophyll concentration of the LL plants. Tissue hydration was not affected by the light treatments. Values that are significantly different are indicated in Table 1.

DISCUSSION

The results indicate that light intensity is a critical factor for the survival of Texas madrone seedlings. This suggestion is further substantiated by our observation (unpublished) that madrone seedlings that survive under natural conditions

usually are found growing where the light intensity is about 6,500 to 10,000 lux. Well-established plants tolerate full sunlight, however.

TABLE 1

Effect of Light Intensity on Madrone Seedling Growth and Chlorophyll Content

Light Treatment	Plant Ht cm	Fresh Wt mg	Dry Wt mg	Dry Wt %	Chlorophyll mg/ tissue
17,200 lux	3.3 ± 0.4	154 ± 6	45.7[1] ± 2.0	31	1.36[1] ± 0.08
12,900 lux	4.0 ± 0.3	165 ± 5	51.0[1] ± 1.5	31	1.52[1] ± 0.07
8,600 lux	4.9[1] ± 0.4	273[1] ± 22	82.3[1] ± 6.1	30	1.82[1] ± 0.12

Each mean (± 2X standard error) is the average of 4 replications.
[1] Significantly different at the 0.05 level.

Because tissue hydration was not affected by the light treatments, we assume that the reduction in chlorophyll concentration caused by the HL and ML treatments is the results of photobleaching. The photobleached plants would have a reduced photosynthetic capacity, and a result, they would tend to be stunted and less vigorous. Thus seedling survival would directly depend upon the ability of the plant to produce the necessary photosynthates.

The data do not rule out the possibility of an interrelationship between light intensity and water availability, however. Although plants of the HL and ML treatments never wilted, the growth medium of these plants tended to dry more rapidly than that of the LL plants. It may be that the seedlings can tolerate a higher light intensity if moisture conditions in the soil are favorable. Such a possibility is currently being investigated and will be discussed in a forthcoming paper.

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POLITICAL PARTICIPATION AND INFLUENCE OF THE AGED

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INTRODUCTION

The aged in America seem to have become qua age a group toward whom political appeals are addressed. The effects of age on political attitudes and beliefs, the efficacy of the aged in utilizing the political process in their behalf, their amenability to utilization as carrier movements for generating support for politically based social and economic developments, all raise significant questions about which there is a variety of opinion. The objective of this paper is to review research and opinion relating to several parameters of the aged as a political force. These are: 1) the desirability of participation by the aged in the political process; 2) political affiliation and attachment; 3) aging and political office; 4) effect of age on political belief; 5) aging-interest organizations and political power.

Desirability of Participation by the Aged in the Political Process

Viewpoints on the desirability of participation by the aged in the political process range from maxims which equate age with wisdom and statesmanship to those that link age with senility and atavistic conservatism. Aristotle, for example, believed that old men tend to turn from the economics of success to the politics of justice. Davis (1953) has suggested that a "chief consolation" of old age is the relative freedom it provides from instrumental constraints and a fuller adherence to principles of conscience.[1]

Parsons (1959) suggests that older persons have a tendency toward altruistic judgments rather than self-interest because "their career commitments have been made, their levels of success are already determined, their decisions are not going to affect their personal fate." Linden and Courtney (1953) similarly suggest that in later maturity the individual naturally becomes concerned with improvement and maintenance of the whole society for the benefit of oncoming generations. Dietz (1968) similarly contends that statesmanship is more likely to be an attribute of age than of youth.

Opposite opinions are found with equal frequency. Pinner (1959) has observed the presence of a concern that "the vision of a future containing a great

[1] see Notes Section of article, page 15.

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many idle, dependent people suggest... vague fears that this group will lay rude hands on the democratic process." Stewart (1970) takes the position that the "old having no future, are dangerously free from the consequence of their own political acts, and it makes no sense to allow the vote to someone who actuarially is unlikely to survive."

Hardly less subtle are the charges that the age of politicians hinders a fresh approach. Butler and Fries (1971) have pointed out that the use of age as a weapon against political figures is often a function of the observer's ideology. They observe that in reality elder politicians are frequently among the most liberal, while younger Congressional members are often pronounced conservatives (or, to follow Adorno's concept of the "authoritarian personality," more anti-democratic, authoritarian, and fascist in outlook).

In addition to the prejudice faced by aged statesmen when their political views differ from those of observers, the matter of jealousy because the elders may be seen as blocks to positions that the observer himself would like to acquire occurs in politics as well as in other areas of life. As Butler and Fries express it (1971):

> ...to younger congressmen who see their careers circumscribed by the holding power of their elders, the problem is a classic one of handing down authority..."Stubbornness and penicillin hold the aged above" wrote former Senator Eugene McCarthy in a poem about old age...

It was the same process that prompted James Fazy to similarly remark regarding the age structure in public office in France in the 1820s in his pamphlet *On Gerontology, or the Abuse of the Wisdom of Old Men in the Government of France*. Fazy said that elder magistrates had "reduced France to...asthmatic, gouty, paralytic, eligible candidates for enfeebled faculties" (Coser, 1971). Some have suggested that anger toward the aged may escalate in the United States in the 1980s as the proportion of power positions in the judiciary, legislative, business and professional areas are occupied by older people, as the number of military and social security retirees increases, and as the tax burden is recognized to fall with increasing weight on the middle-aged taxpayer (Neugarten, 1974).

Opinions regarding the suitability of active participation of the aged in the political process thus range from the view that sees the aged a peculiarly suited to taking a long-range and altruistic perspective to a view which sees the aged as inherently shortsighted and as a menace to a democratic system. Furthermore, the status of the aged may function as a convenient and visable club with which to impeach an alien ideological perspective.

Political Affiliation and Attachment

Individuals who may seriously consider actively participating in a campaign may nevertheless develop a strong psychological attachment to a set of principles, a party, or a candidate. Studies have consistently shown that older persons as a

group are more likely to be strongly attached to a major political party, whereas younger persons are more likely to claim to be nonpartisan or independent. Campbell (1971) suggests that greater party identification among the aged is only indirectly a function of age. The critical factor seems to be the length of time they have identified with a party. The stronger attachment among the aged is due to the fact that aging provides the opportunity for longer-standing identification.

Campbell (1971) found it particularly interesting that George Wallace drew a larger proportion of votes from *under* 35 voters than he did from older cohorts. The popular image that Wallace's emphasis on domestic "law and order" and integrity in foreign affairs appealed disproportionately to older people seems to have been at least partially due to a cultural persistence of aggregates (perhaps aided and abetted by the social sciences) wherein age was pejoratively associated with dogmatic, nonlogical, reactionary, or traditionalistic principles. Irrespective of the publicized turnout of collegians in support of Senators McCarthy and Kennedy, survey data reveal that in the 1968 presidential elections the collegians were swamped at the polls by their own age mates who provided the impetus of support for a populist third party thrust (Gulliver and Murphy, 1971). Lipset and Ladd (1971) have noted that over 25% of the newly franchised 18–21 age group not in college preferred Wallace to either Muskie or Nixon.

Age of Office Holders

There has been a long-term increase in the age at which United States citizens are most likely to serve in legislative, judicial, and diplomatic posts. Individuals are most likely to serve as president, cabinet member, or ambassador between 55 and 59, with decreasing but substantial proportions continuing to hold office beyond age 60 (Schmidhauser, 1968).[2]

The average age of Congressional members at the start of the 88th Congress was 52.7; that of the 89th was 51.9. The average of senators (which ranged from 32 to 87) rose from 56.8 to 57.7 years, but dropped from 51.7 to 50.6 for representatives. House members ranged from 25 to 87 years of age. Senators have long been a relatively senior group at the Congressional level. The average age of senators in 1900 and in 1962 was the late fifties. Average age of house members increased from 58 to 63. In the November 1970 elections 12 Congressmen with an average age of 61 (and with an average of 15 years seniority) were defeated by challengers with an average age of 45.5.

United States Supreme Court Justices are typically appointed between the ages of 55 and 59.27. One analysis of Supreme Court and Federal Courts of Appeal judges indicated that the upward trend in age of selection until 1932 may have been followed by a slight downward trend during 1933–1961. Calling and Leidecker (1971) have shown that the termination age for Supreme Court places has decreased steadily since 1900 and seems to have been saturated in the low 60s.

[2] see Notes Section of article, page 15.

The presence of aged persons in positions of leadership is not necessarily indicative of the viability of the aged individually or collectively. Interpretation of political races in which a "young" candidates themselves. Almost inevitably when a person whose youth is so disparate as to be regarded as an attention-drawing characteristic achieves political office, it is often largely the result of circumstances over which the beneficiary exerted little positive control. The 25 year old Congressman, for example, or the 32 year old governor, is likely to be the beneficiary of a father who occupied a similar position himself, or who at least inherited above-average capital resources along invaluable enabling perquisite. Even on the local level, inheritance of family finances, contacts, reputation, property control, professional practice, geographical community stability, all place the fortunate youth many rungs ahead.[3]

Effect of Age on Political Belief and Practice

The belief that people tend to become more conservative as they grow older is conventional if inaccurate wisdom (Glenn, 1974). Pioneer sociologist Charles H. Cooley (1962) claimed that the old, in contrast to the bold and innovative temperament of the young, are quiet, timid, and conservative. W.I. Thomas (1966) another classic sociologist, described the youth of a community as more likely to develop new attitudes and values. Hollmon (1971), noting a decennial increase in the proportion aged persons in the population of a southern state, suggested that tendencies toward progressivism might thereby be overwhelmed by stagnation and conservative attitudes.

Sociologist George Lundberg asserted that "...radicalism is the attitude of the young, dynamic society; conservatism the attitude of the old comparatively static society." Landis (1948) claimed: "...a certain amount of inertia accumulates with experience." Bernard (1957) concurs that "...even with the same amount of education, older persons tend to be more conservative than younger persons."

Fengler and Wood (1972), in a comparison of 3 generations, found that each generation was more liberal than the one which preceded it. Glamer (1972), in a study of "conservatism" relative to law enforcement, race relations, and patriotism, found age to be a less important variable than education. While age was positively related to conservative opinions, Glamer felt this was the result of the liberalism of the younger group rather than increased conservatism among the aged. The modal classification for the aged respondents was in the moderate category, while younger respondent's were skewed toward the liberal end of the scale. Both the younger and older cohorts had their conservatives, but the younger group had many more liberals.

Glenn (1974) contends that cohort analysis of ample survey data in the United States in the last 3 decades justifies two general conclusions: 1) people have typically become *less* rather than more conservative with age according to any consistent definition of conservatism, thus conforming to societal trends, and

[3] see Notes Section of article, page 16.

2) persons who have aged into and beyond middle adulthood in the last 3 decades have become conservative *relative* to the total adult population and probably relative to prevailing definitions of conservatism and liberalism. While aging populations have changed in the same direction as the total population, those aging beyond adulthood have generally changed much less than the total population. This increase in the relative conservatism of the aging may be reflected in a comparative increase in the conservative identification of aged cohorts vis-a-vis the total environment at the same time the aged cohort has become less conservative relative to a constant definition of conservatism.

Berelson, Lazarsfeld, and Gaudet (1948) concluded from their study of presidential voting in Erie County, Pennsylvania in 1940, that older persons reflected not so much political as social conservatism. The same authors concluded from their voter behavior study of Elmira, New York in the 1948 presidential election that party affiliation was less important in "age generations"in Americanpolitics than are social factors which are basic to allegiance. The generation which came of age in the 1920s tended toward Republicanism, but the underlying split in this generation was religion. The political generation which came of age in the 1930s tended to be more Democratic in affiliation, but the basis for division of socioeconomic.

Three major issues which divided the electorate at the turn of the 1970 decade were a) government welfare programs, b) foreign affairs, and c) civil rights. The 1960 Survey Research Center (SRC) survey asked several questions which tapped the general welfare area (Campbell, 1971). Questions suggesting that the federal government take responsibility for "full" employment and for a minimum level of living for everyone received no significant difference in response by age. A suggestion of federal aid to local elementary and high schools was similarly rejected by all age groups, but persons over age 65 were least likely to approve. However, it was the over-65 respondents who were most approving of a proposal that the federal government aid people to obtain hospital care and physicians' services at low cost.

Widespread social changes in the area of race relations occurred during the 1960-1970 decade which stirred conflicting reactions in the electorate. SRC data indicated white persons over 65 were most likely to be opponents of federal action in enforcement of social rights of blacks in employment, public education, public accomodations, and housing. While there was a similar division of white attitudes at all age levels, there was a greater expression of resistance among older persons.

SRC data gathered in 1968 asked respondents to place themselves on a scale ranging between 2 extreme approaches to "the problem of urban unrest and rioting," of using "all available force to maintain law and order" on the one hand to taking action to "correct the problems of poverty" on the other. Campbell (1971) reports little difference in the way age groups placed themselves on the scale. White people in their 50s and beyond were somewhat more likely to

favor "all available force" than were younger persons. Persons over 65 were much less likely than people in their 20s to feel that the police had used excessive force at the demonstrations at the time of Democratic National Convention in Chicago in 1968.

Campbell feels that "for the most part the age differences in the way people perceive and respond to politics derives from either of 2 sources, from experiences they have during their early period of political socialization, or from their present circumstances.[4] Therefore, it is not surprising that people who learned the pattern of race relations prevailing in the 1920s are likely to have different attitudes toward social rights legislation than were college graduates of the 1960s. A similar explanation may be advanced for the general findings that older people have been found in survey data to be more opposed to government support for social rights conflict, election of a catholic to the presidency, and to more government control in general.

However, when government intervention in the economy can benefit them in particular, old people are at least as likely as younger persons to favor interventive measures. Older people seem especially responsive to government payment of medical costs and efforts to secure jobs. Surveys have indicated that older persons are somewhat more likely to favor public housing and publically owned electrical power. (Campbell, 1971).

Schrieber and Marsden (1972) opined on the basis of survey data that clusters of values associated with class are more important explanatory variables than is age. They found a marked blue-collar/white-collar dichotomy in support of a government program of medical care. The lesser likelihood that white-collar person favored a government program of medical aid was interpreted as falling within the notion of "economic security" in that their accumulated financial security supposedly would be greater. The authors felt, however, that the effect of occupation and age on pro-medical aid opinion could well reflect ideological elements as well as "economic self-interest".

Back and Poss (1972) suggest that age as such may exert little definitive affect on the public opinion of a cohort. To a large extent, differences may be accounted for by such other factors as social class, region, race, or religion. Applying cluster analysis to data collected periodically by the American Institute on Public Opinion, Back and Poss found that there was a tendency for older groups to belong to the conservative group of responses to questions regarding foreign relations. The older were more likely to be anti-communist (particularly in the 1950s as compared with the 1960s data) and isolationist while younger persons were more likely to appear in the anti-war pro-foreign initiative cluster.

The legend that age equals conservatism is not entirely without substance in that a number of studies of social values regarding religious beliefs, behavior norms, public welfare, marriage, divorce, child rearing, etc. have indicated that

[4] see Notes Section of article, page 16.

"older people are considerably more apt to defend traditional positions than are younger people" (Carlie, 1969; Cutler, 1971). Other research, particularly in recent years, has raised considerable challenge to the hypothesis that the aging process involves intrinsic tendencies toward conservatism without major qualifications to any supposed linear relationship (Glenn, 1974).

Campbell (1971) has interpreted the larger proportion of older people favoring a government program of medical aid as a reflection of "economic self-interest" rather than political ideology. Schreiber and Marsden's study (1972) on the relationship of opinion on a government program of medical aid to the aged based on data from 4 random samples of the American adult population showed that persons over 65 were more likely to favor such a program. Support for Campbell's economic self-interest hypothesis was indicated by the fact that the association between age and pro-medical aid sentiment was reversed when income was held constant. Persons with lower incomes were more likely to favor a program of government medical aid than were those with higher incomes regardless of age.

Campbell contends that a more accurate description of the relation of age to attitudes is that changes which take place throughout the life cycle reflect the circumstances that an individual is in at the time. A parent whose children are of school age is thought to feel more disposed toward school taxes, while the retired person is thought to be less inclined to further tax himself for school support.[5] The other side of the supposition is that the retired person is more likely to be favorably inclined to federal underwriting of medical costs despite the fact that such programs have almost been regarded as rampant communism in some segments of the population until very recent times. Campbell holds that the general public, old and young, is far less ideological in its thinking than is generally thought.

The legend that age equals conservatism is not without researchs support. Numerous studies of social values regarding religious belief, behavioral norms, public welfare, marriage, divorce, child rearing, and so on have indicated that "older people are considerably more apt to defend traditional positions than are younger persons" (Carlie, 1969; Cutler, 1971). However, particularly in recent years, students of the life cycle have raised considerable challenge to the hypothesis that the aging process necessarily involves intrinsic tendencies toward conservatism without major qualifications to any alleged linear relationship (Glenn, 1974).

Political Consciousness of the Aged

Trends suggesting a dramatic increase in the proportion of aged Americans has caused attention to be focused on the possibility of gains in "senior power" (Binstock, 1974; Douglass, 1974). The 1974 annual meeting of the American As-

[5] see Notes Section of article, page 16.

sociation for the Advancement of Science, for example, sponsored a symposium on "The 1990s and Beyond: A Gerontocracy?" As Binstock has pointed out, the building of a case that increased numbers of senior citizens will result in their becoming a powerful force in American political life assumes "not only that numbers are an important source of power in American politics, but also that masses of citizens can be led to cohesive political behavior on age-based interests" (Binstock, 1974). The vanguard of militant age-based political movement in the United States was the Townsend movement which began in California in the early 1930s (Abraham, 1963). Townsend, a physician, proposed that every aged citizen be given a public pension of $200 a month. At its peak in 1936 the movement had members in every state and a total membership of some 1 1/2 million persons. The movement eventually played out in the 1940s.[6]

At the present some observers claim to see signs of an increased sensitization to age politics among the elderly.[7] One conceivable impetus may be that of a growing age consciousness due to segregation of the aged into an identifiable group qua age vis-a-vis the community as a whole. Some spokesmen feel there is a resultant development of a subcultural consciousness among the aged *as* aged.

Several studies have documented the observation that membership in voluntary organizations is conducive to a high level of involvement in a variety of participatory activities. Trela (1971), who investigated whether participation in associations of age peers in contrast to groups that mix generations facilitated or retarded political identification and activity among individuals 62 years old and over in a Cleveland suburb, found that for the vast majority (circa 90%) of the study population, level of political interest did not seem to be lower than it had in middle age. While those without secondary ties were least likely to claim moderate or high involvement, they were more likely to profess an increase in political interest over time. Persons with multiple association memberships (having both age graded and non-age graded secondary ties) were most likely to profess high or moderate political interest, but were least likely to claim that their interest had increased since age 50.

Trela felt that social class strongly interacted with patterns of secondary association to predispose people to different levels of involvement in political affairs. Since old age is a marginal status, for members of non-age graded associations it may increase social distance between members and exacerbate the effect of low status. Such persons may be likely to be underdogs on the periphery of the group's social and political life so that social class continues to be an insidious factor in political consciousness and social interaction. Older people are exposed to a variety of political beliefs in comparison to which their own beliefs may appear questionable and incompatible if not hopelessly antiquarian or ridiculous. Given the social and psychological disadvantage of lower-class status, these aged (like lower class people in general) may be unwilling to engage in

[6] see Notes Section of article, page 16.
[7] see Notes Section of article, page 16.

political dialogue and other types of political behaviour. In age-graded associations, on the other hand, where age status may acquire new intergrative meaning which generates beliefs characteristic of a political generation, the interaction system may function to create a sense of community with regard to socio-political attitudes.

If the aged are in fact acquiring the characteristics of a separate subculture as some observers suggest (Packard, 1973; Hochschild, 1973), the decreased integration of the aged in mixed groups would, if Trela's findings identify a process that can be generalized, result in an increased development of attitudes leading to political behavior significantly influenced by age status. Trela observed an absence of linkage of age and activist political orientations in the sense of age related politics among members of associations that effectively mixed generations. This was held to be due to shared political beliefs and expectations which transcended mere age identification. In the absence of mixed generational associations, Trela suggested, the salience of age as a political referent seems to legitimize collective action qua age.

It may be that groups of age peers define social reality vastly different from those that mix generations. Individuals who participate exclusively in old-age groups probably are partially removed from the perspective of other generations. Consequently, it is hardly surprising that age tends to become a significant reference point from which older people can define political issues. While such associations may have no ideology proscribing attitudes and beliefs that should be held in common, immersion in a social environment exclusive of other generations may cause a narrowing of political expectations and provide the organization preconditions which foster involvement in political affairs from the perspectives of aging. For members of groups of age peers, participation in a political organization of the aged is defined as appropriate and, in some cases, necessary activity, while for members of mixed generational groups, political participation on the basis of age may be perceived as illegitimate.

A cohort analysis of political alienation as measured by notions of estrangement and powerlessness[8] based on survey data taking measures for the years 1952, 1960, and 1968, revealed that overall political alienation dropped in the period 1952-1960 and increased substantially, regardless of age group, over the years 1960-1968. Internal analysis indicated that different aged cohorts responded similarly in that a near identical pattern was found for each age group and across all 3 political alienation items. The predominant pattern was found pervasive among all cohort groups (Cutler and Bengston, 1975).[9] These findings were summarized as:

> There is no evidence of a process of aging which produces, or is in other ways related to, political alienation among

[8] see Notes Section of article, page 16.
[9] see Notes Section of article, page 17.

> adults during the period studied. At least for this period of 16 years of aging in the 1950s and 1960s, aging in the younger years of the life cycle did not produce differences in the way in which political alienation changed; rather, the basic pattern found for the total population is that which is descriptive of the component age groups within the population.

The Lorge and Helfant study of sociopolitical verbal attitudes of a group of adolescents and parents indicated that "for the range of adults tested (36-64) there is no evidence of increasing conservatism in the older years" (Newcomb, 1967). Newcomb, in a longitudinal study wherein a sample of undergraduates were followed to middle age, found considerable consistency in political attitudes. Some individuals became more conservative while others gravitated in a more liberal direction. There was no consistent drift pattern in either direction. Such findings cast suspicion on the popular notion that political attitudes move rightward as a concomitant of the physiological changes associated with life-cycle maturation.

Each generation takes off from a different starting point in time, and some residue of this differential doubtless persists. However, it cannot be presumed that people typically move through life-cycle change which carries them across the political spectrum. Campbell (1971) has pointed out that there is almost no systematic evidence--countless anecdotes notwithstanding--of political change over the life cycle. Campbell feels that "the assumption so often expressed that political attitudes move right as a natural aspect of aging offers an appealing analogy with the physiological changes associated with age, but it is probably far too simple to explain what actually happens.

Campbell suggests that the individual citizen of any age reacts to his situation as he sees it. This may lead him to support conservative policies, liberal policies, or, with little sense of conflict, both at the same time. Since each age cohort includes people who profoundly differ in many important circumstances of life, it is not likely that any age group will be particularly homogenous in attitudes. In fact, the evidence which national surveys provides does suggest that attitudinal differences between age groups are far less extensive than those within age groups.

Aging-Interest Organizations and Political Power

The question of the potential power of the aged must necessarily consider the role of aging organizations as they have merged in the last decade. In spite of thinly veiled threats of aroused political consciousness from the aged if desired bensions of "positive government" are not cast their way, most academic observers seem to feel that a political movement by elderly persons based on age as such is currently unlikely. Campbell, for example, takes the position that age is not a very strong force in American politics at the present time and is not likely

to become such in the immediate future. Age groups, he feels, will continue to remain as heterogenous in economic, social, and geographical characteristics as they are now; this heterogeneity will frustrate attempts to make common cause among people who resemble each other only in their age. Young people may develop greater visibility as a pressure group. People over 65 may have thereby learned something about the techniques and payoffs of pressure politics and, if so, might be expected to attempt to extend their effort in this direction. However, "there is no instance in modern history in which a major political movement has grown out of the interests of a particular age group, and it seems impossible that any such development will occur soon."

An early opinion by Holtman (1954) suggested that championship by powerful and respectable proponents of expansion and liberalization of the social security system was an important socio-political role of the aged in the future. A study of San Francisco's aged lead its authors to conclude that their sample of elderly subjects gave "no indication that the aged are 'emerging' in the form of a minority movement...it is nowhere evident that they are functioning as a unified group with indigenous leadership, focused political power, and shared long-range aspiration...they have never agitated for the elimination of their second-class status (Clark and Gallatin, 1967).

Binstock and Lohman (1971) note that while most politicians wish to avoid offending the aged, and many favor proposals to provide incremental benefits to older persons, few are disposed to develop special appeals to the aged among the central issues of their campaign. They regard the partisan allegiance of the aged to be among the most stable within the American electorate, not likely to be shifted substantially by special appeals. Despite the influences of cohort socialization, owing to their geographic heterogeneity, social, and economic differences among themselves, the political attitudes of aged voters and their partisan attachments are diverse. As Campbell (1971) expressed it:

> ...because each age cohort includes people who differ profoundly in many important conditions of life, it is not likely that any age group will be very homogeneous in its attitudes. The evidence which national surveys provide us does in fact demonstrate that attitudinal differences between age groups are far less impressive than those within age groups.

One recurring theme in the case of the aged is the difficulty of establishing a cohesive identity among older persons for political purposes. It may be that aged persons are less likely to think of themselves along age-group cleavages, since it would be derogatory to them due to low evaluation assigned to age in the culture, than are other age groups. This argument maintains that those better off among the older population neither identify themselves as aged nor identify with the problems of the disadvantaged aged.

Most earlier analyses (Schmidhauser, 1968; Cottrell, 1960) and some more recent ones (Greason, 1971) maintain that the influence of aging-interest pres-

sure groups is marginal at best. Carlie (1969) has noted that effective pressure for enacting legislation beneficial for the aged has come primarily from non age-based organizations.

Other observers, to the contrary, feel that since 1965 aging interests have successfully exerted political pressure (Binstock, 1972; Pratt, 1974). One example is that of Medicare, in which the National Council of Senior Citizens, originally organized to support the Democratic nominee in the 1960 presidential campaign (Sundguist, 1968), effectively campaigned for passage of legislation. Aging interest groups have joined hands with the Senate Special Committee on Aging to urge particular policies or interpretations on agencies (Vinyard, 1972). An increasingly benign climate of national opinion on aging questions, as well as the increased lobbying and managerial skills of the organizations themselves, are enabling aging-interest organizations to make maximum use of their increasing resources (Pratt, 1972; Hudson, 1972).

An hostile and unreceptive political atmosphere was a basic feature of the social environment of aging groups in the Townsend era. Rather than serving as staging areas for radical propositions today, contemporary aging organizations are accepted middlemen between government funding sources such as the Administration on Aging and program recipients.[10] 1973 amendments to the Older Americans Act resulted in significant funding increases as well as the development of a new tier of planning agencies (called area agencies on aging) within the states (Hudson, 1973, 1974; Gold, 1974).

Binstock (1974) observes that whether aging-based political orientations result in greater age-interest political clout by the aged depends upon leadership to cohere any existent potential for electoral power or for other forms of political influence based upon a large category of citizens. While hundreds of organizations participate in the politics of national decisions affecting the aged, some 10 organizations built primarily on aging-based interests are currently active in national politics (Binstock, 1972; Pratt, 1974). The post-1960 era has seen an unprecedented outpouring of federal law, executive orders, and new appropriations ostensibly and perhaps to a debatable extent actually directed to the benefit of the aged, all of which have resulted in increases by aging-interest entities at all levels (definitely including state and local) of government.[11]

To some extent the question can be raised as to whether aging interest groups gains in terms of the new organizations which are middlemen brokers largely financed by governmental service contracts instead of membership affiliation represent gains to the aged as such[12]. Although there is definitely a spill-over benefit to the aged in services delivered (nutritional programs, identification cards, sandbox or time-and-interest displacement activities, some advocacy in obtaining benefits of various sorts, etc.)--how much is in reality primarily beneficial to the interest groups who define the aged as their clientel and who

[10] see Notes Section of article, page 17.
[11] see Notes Section of article, page 17.
[12] see Notes Section of article, page 17.

seek to mobilize "senior power" to further agency development. The nursing home industry, for example, receives 3/4 of its income from government, earning high profits for itself while providing less than desirable patient care (Mendelson and Hapgood, 1974).

Since the initiation of the Federal-State programs reflected in the Economic Opportunity Act (1964) and the Older Americans Act (1965) and subsequent legislation (such as the Appalachian Regional Commission or the Law Enforcement Assistant Act), numerous local entities have received their impetus from federal legislation. These entities are able to mobilize considerable local political clout in the furtherance of their vested interests (the Headstart and poverty lobbies are good examples). In addition to the impetus to their formation at the local and state government levels, once organized they have derived much of their promotional, technical assistance, material, and other resources necessary for survival basically from federal funds. State Offices on Aging, created in response to the Older Americans Act, which serve as conduits for dispersement of funds, information, technical assistance, and mobilization of senior citizens to support desired legislative developments and policies, themselves factor into the political clout of the aged. Consequently, political mobilization of the aged is not necessarily imposed from outside of the political process itself. The potential for "senior power" is to some degree governmentally contrived to the extent it is fostered by the organizing, mobilizing and information disseminating efforts of State offices on aging. Within the past decade a significant mobilizing and support force with potential local grass-roots constituency appeal has been extra-locally developed.

Atchley (1972) notes that there "never has been an instance in the history of the United States in which the leadership of an old age interest group could deliver what would even approach 'the old folks vote'." Older people, Atchley feels, are among the least likely to desert long-term party affiliation to vote on specific issues. Atchley is of the opinion that a voting identity similar to the group identity which has created voting blocks among Catholics or blacks is not likely to develop among the older population because the latter is divided in loyalty among many interests groups which supercede the age factor. Pratt (1974), by contrast, notes that by "contrasting their membership derived resources by subsidies from outside sources and by focusing their energies through efficient and goal-oriented actions, aging-interest bureaucracies are having an impact on the national political scene".

Perhaps the most important general assertion that can be made about the contemporary electoral power of the aged is that there is no "aging vote" (Binstock, 1974). There is no evidence to indicate that aging-based interest appeals can swing a block of older persons' votes from one party candidate to another. There is some indication that the aged, at least to date, have not developed a consciousness of age-related problems (Riley and Foner, 1968). There is some evidence that aging voters may respond more homogeneously to state and local

than to national political issues (Henretta, 1973). However, fear regarding an assurance of continued social security income by the soon-to-be and the retired (and perhaps by those wage-earners who felt they would have to make up the losses to individuals) was a major factor in the defeat of Barry Goldwater in 1964, indicating the possible viability of issues of particular salience to aging in a national election (Whitaker, 1976).

SUMMARY OBSERVATIONS

The actual and potential role of the aged in American political life and the effect of the aging process on political belief and behavior are important but debatable issues in the forging of social science policy as well as in terms of social science inquiry. This paper has sought to lift out some issues which revolve around the broader question of the participation of the aged in the political process. The following propositional statements relative to the role of the aged in American political life would seem to be justified:

1. Party attachment seems to be positively associated with increasing age; younger persons seem more disposed as a group to support alternative or nonparty candidates.
2. Among active politicians long-term aging seems more pronounced at the judicial than at the legislative level.
3. There is a contradictory body of opinion as to the suitability, not to mention the desirability, of the participation of aged persons in the political process.
4. The common sense mythology which equates age with conservatism in political outlook has received some concurring research support; however, contradictory research findings as well as casual observation is adequate to contradict the popular notion of a simple linear relationship between aging and increased conservatism in political opinion.
5. Cutting across the contradictory impressionistic and research data on the generally supposed relationship between age and heightened conservatism are indications that while aged cohorts become *less* rather than more conservative relative to a constant definition of conservatism, they possibly become more conservative relative to the total population.
6. To the extent that there exists a correlation between age and conservative political opinion the relationship may be largely a reflection of a more fundamental link between age and social conservatism. Furthermore, age may be less important as a determinant of political opinion than are other factors such as race, religion, region, or social class.
7. Some observers feel that the political impact of the aged may be increased by the development of a distinct subculture among the aged that will facilitate the development of a political consciousness and lead to an increased viability of "senior power".
8. Some observers feel that the potential impact of the aged as an organized group is minimal because most of the aged have developed loyalties to groups which cut across the political spectrum.

Examination of the political role of the aged in the future must necessarily take into account the role of "middlemen agencies and programs" which have been created to service the needs and interests of the aged as well as the training of personnel to staff the institutional and organizational positions and to coordinate and supervise the various programs conducted on behalf of the aged. Sternlieb (1971) has noted "two levels of reality" in the demand for expanded public services and functions to meet the needs of the aged. One level is the actual effort to promote the interest as claimed. Beneath this is a second level of reality--that of who gets the contract, the grant, the patronage, the potential positions in the service agencies.

One might speculate that as the size of the public sector devoted to servicing the needs and interests of the aged increases, politics and political controversy in the interest of the aged, particularly in the expansion of agencies for whom the aged are the primary clientel, will take on a corresponding increase in importance. It may be that in the future the political clout of the aged may be bolstered less by the aged acting in concert for themselves as by agencies seeking to bolster and expand services which they provide to the aged. Such a development would seem to be a factor which must be taken into consideration in the future.

NOTES

1. Davis' illustration was in terms of freedom from individual constraints which restrain conscience.

> It is quite true that 'tis man's perdition to be safe when for the sake of truth he ought to...lose his job, with small chance of getting another. But it is, emotionally if not ethically, a somewhat different matter to tell a young man with a wife and children when he is barely able to support them on his salary that, for the truth, his wife and children ought to starve too.

One might suggest, however, that these constraints may really bear most heavily on the middle-aged in the sense of diminished opportunity for re-establishment, particularly in terms of employer prejudice. Davis' conclusions are otherwise challengeable too; they are given only as illustrative of a spectrum of opinion, nor as fact nor principle.

2. A person who has to achieve the base of experience, education, residential stability, and income from a standing or self-generated start is practically disfranchised from becoming successful in elective political life in a comparative sense. At the younger ages he is disfranchised because it takes time for him to get into the arena. A contest for political office in which a 25-year old is pitted against a 50-year old may be a race in which the 50-year old novice is really the "youthful" candidate in that he may be running against several generations which culminated in the opportunity for the 25-year old to make the race. To disfranchise the acceptability of the former *because* of age would represent that much of a decline in democratic opportunity to occupy elective position through one's *own* interest, effort, chance, and vitality. The extent to which congressional seats and executive chairs are occupied by persons whose positions are obtained through inherited resources raises serious questions as to the effective dispersion of political control and opportunity (unsigned, 1976).

3. The visible presence of aged persons in positions of political leadership is not necessarily indicative of the viability of the aged individually nor collectively. Interpretation of political races in which "young" candidate is pitted against an "older" candidate in terms of the chronological age of the candidates themselves may be misleading. Almost inevitably when a person whose youth is so disparite as to be an attention-drawing characteristic achieves political office, it is largely the result of circumstances over which the beneficiary exerted little positive control. The 25-year old congressman, for example, or the 32-year old governor, is likely to be the beneficiary of a father who either occupied a similar position himself or who otherwise received above average capital resources and other enabling perquisites. Even on a local level, inheritance of family finances, contacts, reputation, education, professional practice, property, geographical community stability, may place the fortunate youth many rungs ahead. The 50-year old in a given political race against a particular 25-year old may be opposing, not merely a chronological junior, but a generational lineage.

4. Such findings are consistent with Binstock's observation that if the disadvantaged aged see their economic, housing, nutritional, recreational, or transportation problems as aged, they see them in other contexts as well. "A full life cycle of socialization, experiences, and attachments--family, schooling, ethnicity, occupation, income, residence, peer and other associations...it would not be unreasonable to infer that many of those who identify as aged--even those responsive to the special needs of the disadvantaged aged--would have stronger competing identifications and interests" which substantially dilute attempts to mobilize a cohesive voting bloc (Binstock, 1974).

5. Such reasoning, however, is hardly an explanation. Equally appropriate reasoning could be given to explain why older persons might be more likely than parents to support school funding. These might include such reasoning as the parent with small children may be less able to afford increased expenditures, or being more intimately knowledgeable about legitimate school needs, may feel that increased taxation for school purposes is not appropriate, and a host of possible reasons.

6. Pratt (1974) notes that even in California the Townsend plan was acceptable to less than 5% of the population. The plan was vigorously condemned by the Roosevelt Administration and met with hostility by the House Ways and Means Committee. Current age-interest organizations, by contrast, particularly those which act as conduits for dispersal of funds and administering of technical assistance through government financing, are both nurtured and supported by government. Elected officials, since the Kennedy-Johnson era, Pratt feels, seem to have accepted the view that spokesmen for senior organizations not only merit hearing when they speak, but that they should be encouraged to voice their policy preferences.

7. The Texas Senior Citizens Association, for example, which grew out of a social club for "retirees" in Port Arthur, Texas in the mid 1960s, claims to have been instrumental in securing such favorably discriminatory treatment for the aged as passage of a Homestead Bill and reduced bus fare. The main purpose of the organization, according to its constitution, is "to unite the efforts of retired people and people living on 'fixed' incomes to enable them to wield a fitting influence in local, state, and national affairs." Its founder-president is registered and active as a legislative lobbyist. The White House Conference on Aging in 1971 is said to have stirred considerable impetus for the aged to flex their political muscle, with sponsorship of "senior power" a noticeable objective (unsigned, 1971). Urging of oldster's to take a legislative initiative seemed to have been influential in getting attention to "concerns of seniors" (unsigned, 1976).

8. Alienation as thus conceived was measured by response to the questions of 1) "I don't think public officials care much about what people like me think;" 2) "People like me don't have no say about what the government does;" and 3) "Politics and government seem so complicated that a person like me can't really understand what is going on." These,

taken together, were held to indicate "both the estrangement which the individual feels toward government and the powerlessness he feels to modify or change the situation" (Cutler and Bengston, 1974).

9. See Cutler and Kaufman (1973) and Evan (1965) for findings which point in a similar direction: aging cohorts generally follow societal trends, although younger cohorts may show somewhat more change than older cohorts.

10. A particularly lucrative statuatory basis for many of these middlemen programs has been the 1965 Older Americans Act under which the national government distributes over $20 million annually. Organizations such as the National Council of Senior Citizens, for example, were effective in pressuring the OEO to create a task force on the "special problems of the elderly poor," which when established consisted largely of the NCSC and its allies. Their interests were given favored treatment in the subsequent subletting of OEO contracts (Binstock, 1972).

11. State offices, generally controlled by the State Chief Executive, serve as conduits for federal funding agencies as well as mobilizing support on State issues "by providing regular information and technical assistance on issues affecting the elderly" (unsigned, 1976), organizing Governor's Conferences on Aging, publishing a periodical which provides inspirational, programmatic, and legislative news, and other activities conducted by the State organizations.

12. See Pratt (1974) for discussion of the application of performance criteria, in which age confers no special advantage, among bureaucratized aging-interest groups.

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Notes Section

THE MEDITERRANEAN GECKO, *HEMIDACTYLUS TURCICUS*, AT BIG BEND NATIONAL PARK, TEXAS—Since its original establishment at Brownsville, the Mediterranean gecko, *Hemidactylus turcicus*, has extended its range in Texas westward to Del Rio and north to Denton (Raun and Gehlbach, *Amphibians and Reptiles in Texas,* Bull. 2, Dallas Mus. Nat. Hist., p. 16, 1972). Recently it has established itself at Langtry, Val Verde County, Texas (Bill Chamberlain, pers. comm.).

At 9:30 P.M. on the night of 10 September 1973, Drs. Timothy and Patricia Brown discovered 2 adult *H. turcicus* On a building wall at Rio Grande Village, Big Bend National Park, Brewster County, Texas. These geckos were not disturbed. During June, 1974, the author visited at night the same building and others in the area, but was unsuccessful in finding *H. turcicus*. However, during July, 1974, on warm humid nights after heavy rainfall during the day, I was successful in finding *H. turcicus* at the one building; all other buildings checked in the area for *H. turcicus* were negative. At 12:45 A.M. on the night of 19 July 1974 I observed 2 *H. turcicus*, 1 adult male and a juvenile about 1/3 grown. The adult was captured, photographed, and released at the site. On the night of 28 July 1974 at 11:45 P.M. I observed 4, 2 adults and 2 juveniles (1 juvenile about 1/3 grown and the other about 1/2 grown). At midnight on 30-31 July 1974 the larger juvenile was again observed.

Hemidactylus turcicus was previously unreported from the park and Brewster County, and the site represents the westernmost record in Texas. It seems evident that the species has recently reached the park, and the presence of juveniles indicates a tiny established breeding population. Although an exotic, the fact that the *Hemidactylus* niche is not occupied by any native species at Big Bend is in the gecko's favor (not competing with any native species). If left undisturbed, in the near future *H. turcicus* will probably establish itself at several suitable localities in the park along the Rio Grande. The origin of the Rio Grande Village population is unknown. The species is known from across the Rio Grande at Boquillas, Coahuila, Mexico, where it has been a resident for at least 8 yrs. (George Burdick, pers. comm.). Another likely source of origin is the constant flow of vehicles into Rio Grande Village. Many trucks visit the Rio Grande Village store, and the accidental stowaway and transportation of these geckos into the park in such trucks seems very possible.

Appreciation is extended to Dr. Timothy W. Brown for sharing his knowledge of the Big Bend herpetofauna. Color slide photographs of the male *H. turcicus* are deposited in the Big Bend National Park slide collection and the Northwest Missouri State University vertebrate collection.—*David A. Easterla, Department of Biology, Northwest Missouri State University, Maryville 64468.*

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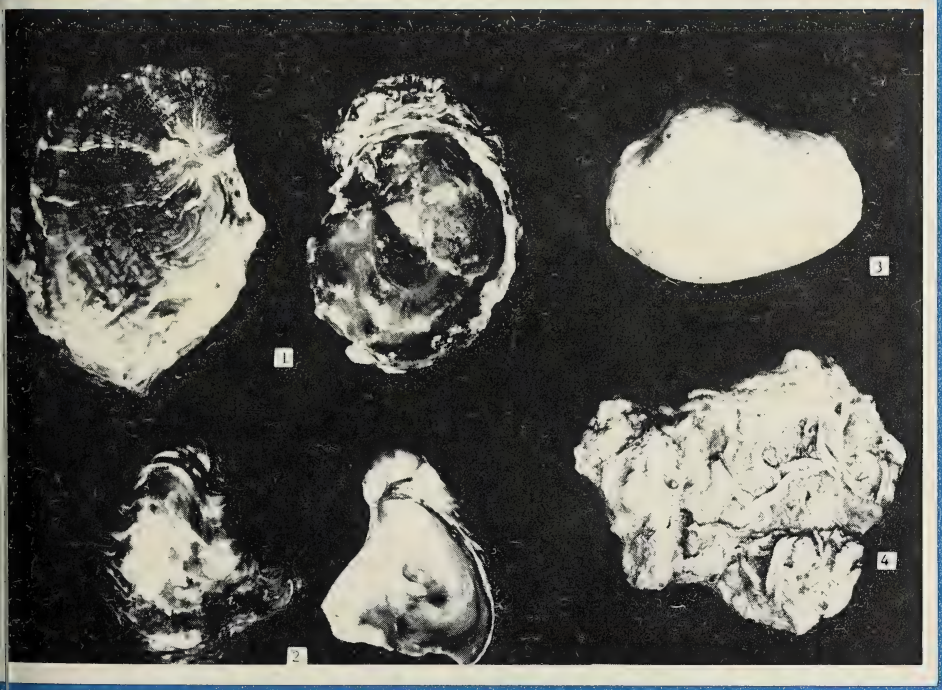

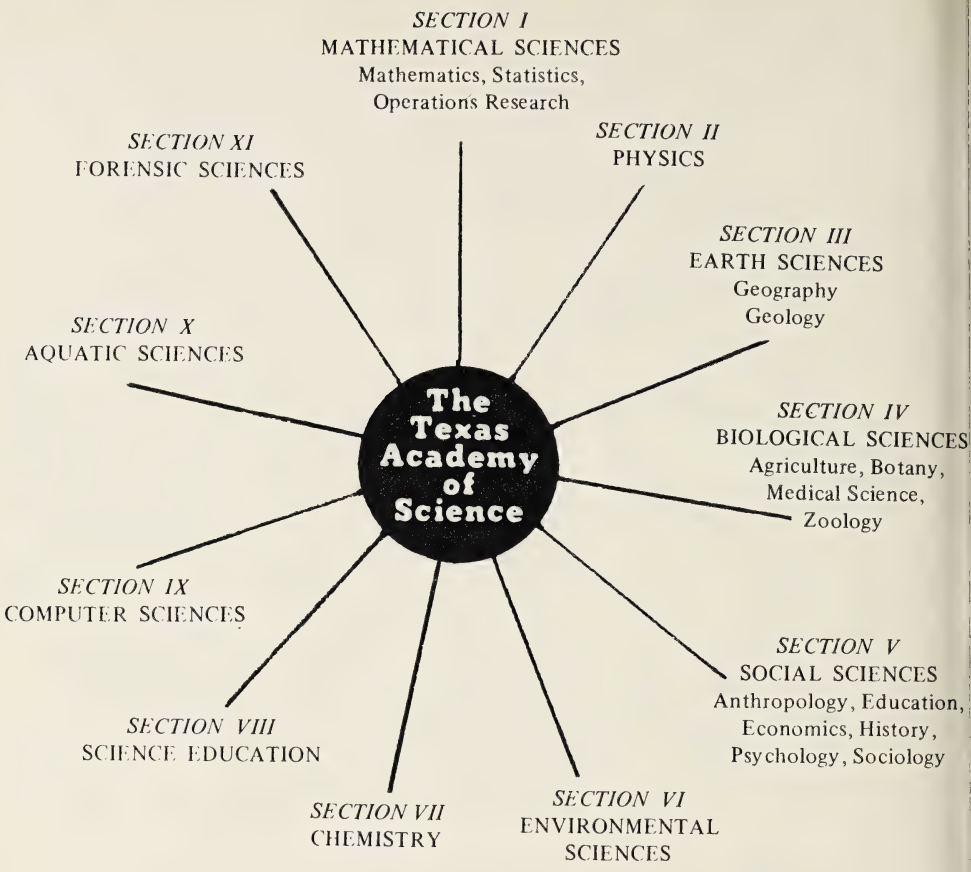

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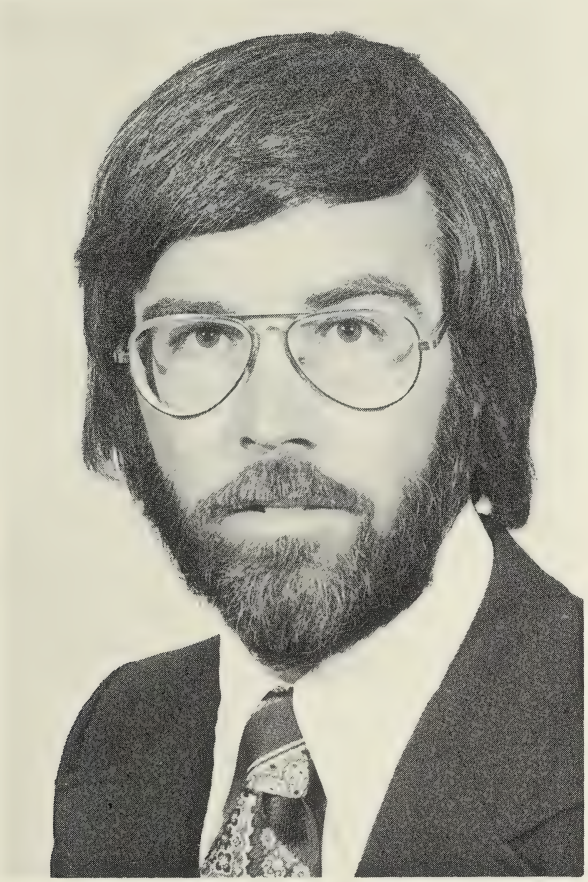

ROBERT W. GRACY, PH.D.

BIOGRAPHICAL SKETCH

Robert W. Gracy received his Ph.D. in Biochemistry from the University of California, Riverside. While there he was a Fellow in the laboratory of Ernst Noltmann. He then joined B. L. Horecker as a Fellow of the Damon Runyon Cancer Foundation at the Albert Einstein College of Medicine in New York. Subsequently, as a Senior Investigator of the Alexander von Humboldt Foundation, he worked with Ernst Helmreich at the University of Würzburg, West Germany. He has been the recipient of a Research Career Development Award of the National Institutes of Health, and is currently Professor and Chairman of the Department of Biochemistry, North Texas State University Health Science Center and Texas College of Osteopathic Medicine, where he has been a member of the faculty since 1970.

THE QUANTITATIVE MEASUREMENT OF METHYLGLYOXAL SYNTHASE IN TISSUE AND CELL EXTRACTS, THE DISTRIBUTION OF THE ENZYME

by P.M. YUAN, G.E. BRENTON, P.K. TSAI, *and* R.W. GRACY[1]

Department of Chemistry,
North Texas State University,

and Department of Biochemistry,
Texas College of Osteopathic Medicine,
Denton 76203

ABSTRACT

A highly sensitive, radioisotope assay has been developed for methylglyoxal synthase. This and 2 additional methods were compared and shown to provide valid procedures for the quantitative measurement of the enzyme in crude cell extracts and tissues. These studies showed that interference by triosephosphate isomerase or other enzymes, which also utilize dihydroxyacetone phosphate as substrate, is insignificant in cell extracts and tissues. Homogenates of human and rat tissues revealed no traces of methylglyoxal synthase. Similarly, a variety of vertebrates, invertebrates, yeast, fungi and algae failed to exhibit the enzyme. On the other hand, a variety of Enterobacteria and closely related species contained the enzyme and exhibited specific activities ranging from 0.02 to 0.5 units/ mg of protein.

INTRODUCTION

Methylglyoxal synthase [dihydroxyacetone phosphate phospholyase (EC 4.2.99.11)] catalyzes an internal oxidation reduction and phosphorolysis of dihydroxyacetone phosphate to methylglyoxal and inorganic phosphate. The enzyme constitutes the 1st step of a glycolytic bypass from dihydroxyacetone phosphate to pyruvate which may be regulated by inorganic phosphate (Cooper and Anderson, 1970; Hopper and Cooper, 1971). The synthase has been purified from *E. coli* (Hopper and Cooper, 1972), *Pseudomonas saccharophila* (Cooper, 1974) and has been recently isolated in crystalline form from *Proteus vulgaris*

[1] Recipient of a Research Career Development Award from the National Institutes of Health (K04 AM70198) and to whom correspondence should be addressed.

Accepted for publication: December 5, 1977.

(Tsai and Gracy, 1976; Yuan, *et al.*, 1976). The chemical and physical properties of the enzyme have been of interest to this laboratory for some time, and a detailed study of catalytic properties has recently led to a proposed mechanism of catalysis (Yuan and Gracy, 1977).

In spite of the above studies, little is known regarding the distribution of the enzyme. For example, it has not been established if the enzyme is present in both eukaryotic and prokaryotic organisms. Since dihydroxyacetone phosphate can be readily metabolized by several other enzymes such as triosephosphate isomerase, aldolase, and α-glycerophosphate dehydrogenase, it is possible that these competing enzymes may interfere with the detection of methylglyoxal synthase. Furthermore, methylglyoxal synthase is allosterically inhibited by inorganic phosphate. Thus, a number of potential problems exist in the detection and accurate measurement of methylglyoxal synthase in tissue or cell extracts. The present study was undertaken to (a) develop and test several methods for accurately measuring the enzyme in such extracts, and (b) to survey a variety of organisms for the presence of the enzyme.

MATERIALS AND METHODS

Dihydroxyacetone phosphate (dimethylketalcyclohexyl ammonium) was obtained from Sigma and converted to the free ketone according to the instructions of the supplier. Methylglyoxal was distilled prior to use (Friedemann, 1927). Glyoxalase I (EC 4.4.1.5; specific activity 360 units/mg) and reduced glutathione were from Sigma, while ^{14}C-glucose was from New England Nuclear. Radioactive dihydroxyacetone phosphate was synthesized from ^{14}C-glucose as previously described and isolated by ion exchange chromatography (Yuan and Gracy, 1977). Crystalline methylglyoxal synthase was isolated from *Proteus vulgaris* as described previously (Tsai and Gracy, 1976) and exhibited a specific activity of 14 units/mg. Protein was determined by the Coomassie Blue dye-binding colorimetric procedure of Bradford (1976).

Three different methods were utilized for measuring methylglyoxal synthase activity. The 1st procedure involved the glyoxalase I-catalyzed coupling of the product (methylglyoxal) to reduce glutathione. The formation of S-lactoyl glutathione was monitored at 240 nm (Yuan and Gracy, 1977). Measurements were carried out in a recording spectrophotometer at 30C, and the assay mixture contained 50 mM imidazole buffer, pH 7.0, 1.0 mM dihydroxyacetone phosphate, 1.7 mM reduced glutathione and glyoxalase I (1 unit/ml).

Secondly, the enzyme was measured by a colorimetric assay similar to that described by Cooper (1975). The enzyme was incubated in 0.5 ml of 50 mM imidazole buffer, pH 7.0, containing 1.0 mM dihydroxyacetone phosphate. After the appropriate incubation, the sample was mixed with 0.33 ml of 2,4-dinitrophenylhydrazine reagent (0.1%, 2,4-dinitrophenylhydrazine in 2 N HCl) and 0.5 ml of water, incubated at 30C for 15 min, then mixed with 1.67 ml of 10% NaOH. The absorbance at 555 nm was measured after 15 min using a molar extinction coefficient of 4.48×10^4 (Wells, 1966).

The 3rd method developed for measuring the enzyme consisted of a direct isotope assay. ^{14}C-Dihydroxyacetone phosphate was incubated at 30C with the enzyme in 5 ml of 10 mM imidazole buffer, pH 7.0. Aliquots (1.0 ml) were removed at various times and mixed with 0.5 g of AG 1x8 anion exchanger (Bio Rad) to remove the unreacted dihydroxyacetone phosphate and inorganic phosphate. The nonionic product, ^{14}C-methylglyoxal, which was not bound to the ion exchanger, was measured by liquid scintillation counting.

Bacteria were grown to late log phase at 37C in tryptic soy broth. Cells were harvested by centrifugation, resuspended in 2 volumes of imidazole buffer and disrupted by sonication. Yeast and fungi were grown in Sabaroud's media for 36 to 72 hrs to a turbidity of 2.0 absorbance units. The cells were harvested by filtration and disrupted in a tissue grinder with Teflon pestle. Tissues of animals were homogenized in a 2:1 buffer (50 mM imidazole, pH 7.0) to tissue ratio and were assayed immediately or stored at 0 - 3C. Under these conditions the enzyme has been shown to be stable and no loss of activity occurred during storage.

RESULTS AND DISCUSSION

Direct Isotope Assay

Figure 1 shows the relationship of methylglyoxal formation to time when assayed by the direct isotope method. Initial velocities were linear for at least 6 min, but after that time (and especially at higher levels of enzyme), zero order kinetics were no longer followed. When initial velocities were plotted as a function of the enzyme concentration, a linear relationship was observed (Fig. 2), indicating that the rate of formation of ^{14}C-methylglyoxal is a valid indicator of the methylglyoxal synthase concentration.

The rates of methylglyoxal formation as assessed by the direct assay compared favorably with the rates determined by the coupled enzyme spectrophotometric assay or the colorimetric method (Table 1). The colorimetric assay proved to be the most variable and, in general, the least desirable of the 3 methods. These data show that the direct assay is not only useful for studies of the isolated enzyme, but that it is also a valid method for determining levels of methylglyoxal synthase in tissue and cell extracts.

The direct isotope assay method has several distinct advantages over the other 2 methods. First, the level of detection of the enzyme is limited only by the specific radioactivity of the substrate. Thus, the method is much more sensitive than either of the other procedures. Furthermore, the direct isotope method does not require the addition of coupling enzymes, cosubstrates, cofactors, etc., which could influence the reaction. Therefore, the direct assay is not subject to the potential artifacts which may affect the other 2 assay methods.

Assaying Methylglyoxal Synthase in Crude Cell Extracts

Tissue homogenates and cell free extracts of rat liver, skeletal muscle, and brain were assayed for methylglyoxal synthase by the coupled enzyme method.

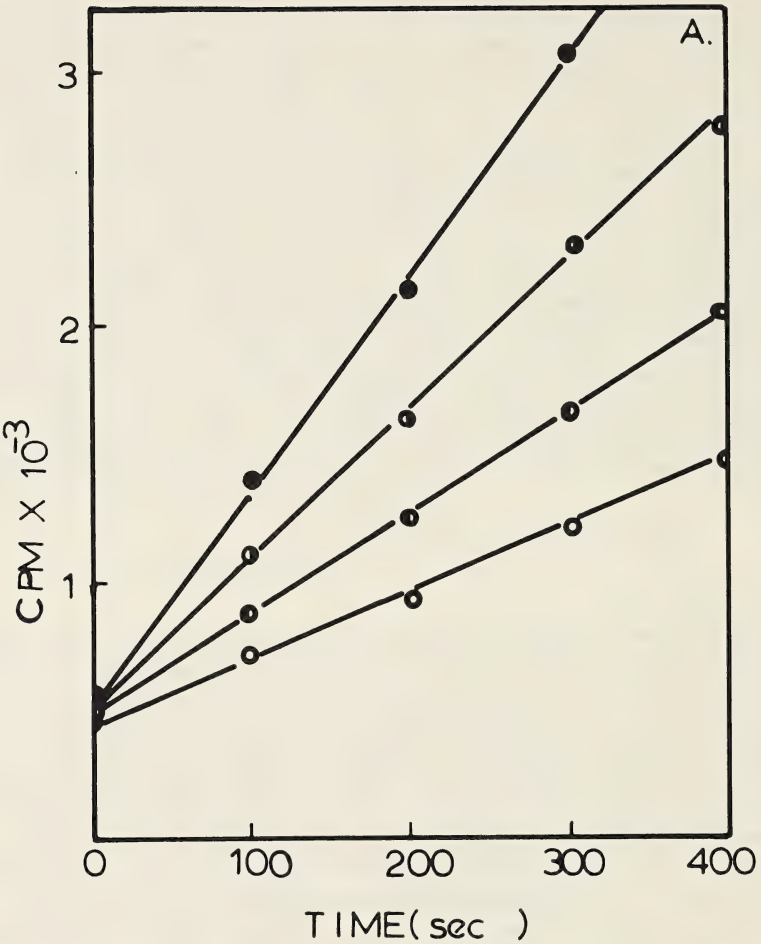

Figure 1. Initial rates of methylglyoxal formation using the direct isotope assay. Methylglyoxal synthase was assayed by incubating ^{14}C-dihydroxyacetone phosphate with the enzyme and counting the radioactive ^{14}C-methylglyoxal formed. Aliquots (0.4 ml) from the Dowex microcolumn effluent were counted in 10 ml of Aquasol (New England Nuclear). The concentration of methylglyoxal synthase for the incubations was 0.0075 units/ml (○); 0.015 units/ml (◐); 0.225 units/ml (◑); and 0.375 units/ml (●).

In all cases, no trace of the synthase was observed. However, it was realized that due to the competing activities of triosephosphate isomerase, aldolase and α-glycerophosphate dehydrogenase, and perhaps other enzymes such as acid and alkaline phosphatases, it might not be possible to accurately measure methylglyoxal synthase in crude cell homogenates.

In view of the fact that the levels of triosephosphate isomerase are so high in cell extracts, this enzyme appeared to be a likely candidate which could deplete

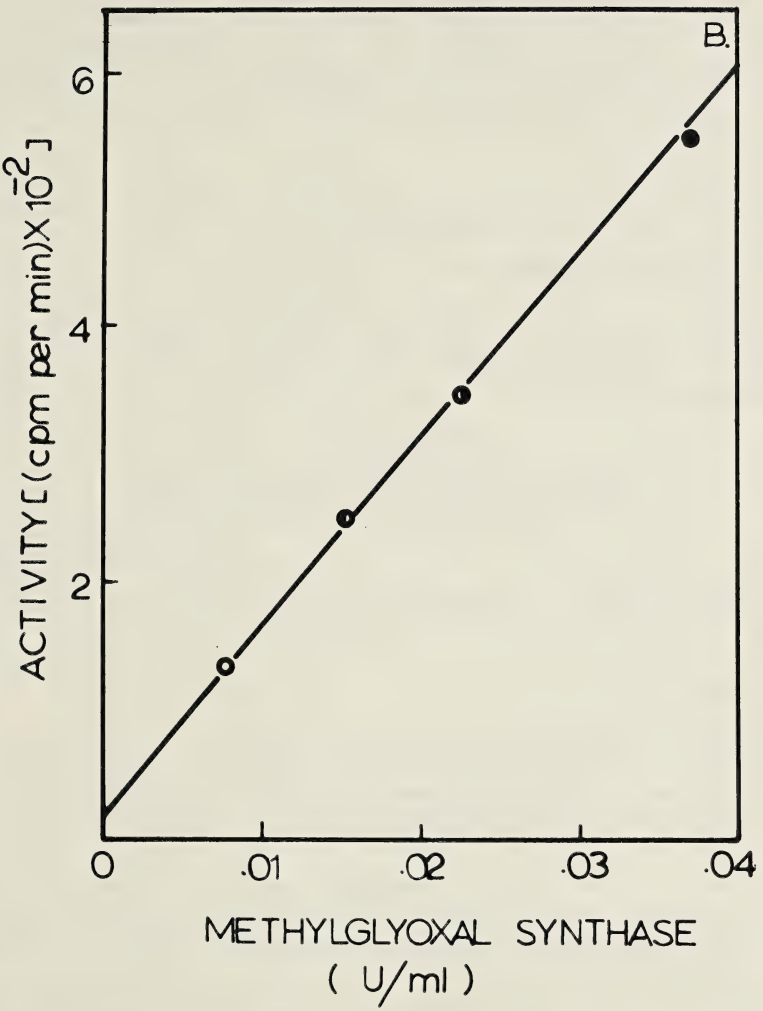

Figure 2. Linearity of initial rates with enzyme concentration. Initial velocities (from Fig. 1) are plotted as a function of the activity of methylglyoxal synthase. The concentration of methylglyoxal synthase for the incubations was 0.0075 units/ml (○); 0.015 units/ml (◐); 0.225 units/ml (◑); and 0.375 units/ml (●).

the substrate. 1-Chloroacetol phosphate has been shown to irreversibly inactivate triosephosphate isomerase by covalently bonding to the catalytic center (Hartman, 1971). On the other hand, this reagent has no effect on methylglyoxal synthase (Yuan and Gracy, 1977). When tissue extracts were incubated with 0.01 mM chloroacetol phosphate at 0 C for 10 min, essentially all of the triosephosphate isomerase was inactivated. As seen in Table 2, inactivation has no effect on the

TABLE 1

Comparison of the 3 Assay Methods for Determining Methylglyoxal Synthase

Assay Method	Activity (units/ml)	
	Pure MGS[a]	Crude MGS[b]
Direct isotope assay	11.2	4.0
Spectrophotometric assay	10.3	3.3
Colorimetry assay	9.0	2.1

[a]Methylglyoxal synthase isolated from *P. vulgaris*.

[b]Crude cell extract of *P. vulgaris*.

measured levels of methylglyoxal synthase. Tissue extracts of the rat showed no detectable methylglyoxal synthase even in extracts in which the isomerase had been inactivated.

TABLE 2

Evaluation of Possible Interference of Triosephosphate Isomerase on the Measurement of Methylglyoxal Synthase

Enzyme Mixture*	Enzyme Activity (units/ml)	
	MGS	TPI
Pure MGS from *Proteus vulgaris*	4.7	0
Rat liver (31.2 mg/ml)	0.0	157
MGS + Liver Extract	4.6	157
MGS + Liver extract + Chloroacetol-P	4.6	0

*Purified methylglyoxal synthase (MGS) was assayed alone or in the presence of triosephosphate isomerase (TPI) in a rat liver extract. Treatment of the mixture with chloroacetol phosphate (0.01 mM) was at 0 C for 10 min.

In order to further test for the possibility of interfering enzymes or inhibitors in the cell extracts, known quantities of pure crystalline methylglyoxal synthase were added to tissue extracts, followed by reassaying the mixture. In all cases, the amount of enzyme measured in the crude extract was as expected. Thus, no interference by competing enzymes or inhibitors was indicated. It, therefore, appears that valid enzyme assays for methylglyoxal synthase can be conducted on tissue and cell extracts.

Human Tissues

Human erythrocytes, lymphocytes, skeletal muscle, kidney, liver, brain and cardiac muscle were tested for methylglyoxal synthase by all 3 methods. In no case was any indication of methylglyoxal synthase observed. Tests were carried out on entire cell homogenates, as well as centrifuged extracts. Cells were extracted in a variety of buffers from pH 6.5 to pH 8.5. Dialysis, the addition of 10% v/v glycerol, or the addition of coenzymes or metal ions had no effect. In all cases,

purified *P. vulgaris* methylglyoxal synthase added to the tissue extracts was 100% catalytically active and showed no inhibitors or interfering enzymes present in the cell extracts. Based on the above, it appears that methylglyoxal synthase is not present in human tissues. The methods employed would have been able to detect as little as 0.01 International Units of the enzyme/gm of tissue.

Other Eukaryots

In addition to human and rat tissues, other species including crayfish, crab, shrimp, Ascaris, snail, lizard, frog, clam, starfish, jellyfish and sponge were examined and showed no traces of methylglyoxal synthase. Liver extracts from lizard (*Anolis carolinensis*) and frog (*Rana catesbeiana*), or extracts of starfish (*Asterias forbesi*) showed a transient increase in absorbance at 240 nm when assayed by the coupled enzyme assay system. However, the absorbance increased only 0.01 to 0.03 over a 2 to 3 min period, then leveled off. The basis of this interference is not certain, but may represent some alpha ketoaldehyde in these extracts which may be enzymatically condensed with glutathione by glyoxalase I. It is interesting that of all of the organisms examined, only these 3 species exhibited this behavior.

Yeast (*Saccharomyces cerevisiae* and *Candida albicans*), algae (*Chlorella sp.*), fungi (*Aspergillus sp.; Trichophyton sp.; Penicillum sp.*) and *Euglena gracilis* (both heterotrophically and photoautotrophically grown) also failed to exhibit the synthase.

Bacteria

Table 3 lists a variety of bacteria found to contain methylglyoxal synthase activity. It appears that the enzyme is restricted to certain enterobacteria. Methylglyoxal synthase is found both in aerobic, anaerobic and facultative anaerobes.

TABLE 3

Comparison of Methylglyoxal Synthase Levels in Bacteria

Bacterium	Methylglyoxal Synthase[a] (Specific Activity)
Aerobacter aerogenes	0.37
Erwinia uredora	(0.055)
Escherichia coli K_{10} (glucose grown)	(0.174)
Escherichia coli K_{10} (pyruvate grown)	(0.190)
Escherichia coli K_{12}	0.50
Escherichia coli B/r	0.14
*Klebsiella pneumonae**	0.26
Proteus vulgaris	0.15 (0.064)
Proteus rettgeri	(0.020)
Salmonella parathyphus	0.47
Serratia marcescens	(0.043)
Shigella boydii	0.39

[a]Values in parentheses are from reference (Cooper, 1974).

In addition to the bacteria listed in Table 3, the following organisms were tested but exhibited negligible activity (i.e., less than 0.0005 units/mg): *Bacillus megaterium; Bacillus subtilis; Clostridium butyricum; Clostridium sporogenes; Corynebacterium diphtheriae; Erwinia carotovora; Lactobacillus bulgaricus; Lactobacillus casei; Leuconostoc sp.; Pediococcus cerevisiae; Pseudomonas aeruginosa; Pseudomonas ovalis; Staphylococcus citres, epidermis* and *lactis;* and *Streptococcus thermophilus. Azotobacter vinlandii* and *Streptococcus faecalis* have been reported to not contain the enzyme (Hopper and Cooper, 1971) and were not reassayed by this laboratory.

It is of interest that the levels of methylglyoxal synthase varied widely (0.02 to 0.50 units/mg). Since it has been postulated that the methylglyoxal shunt is allosterically responsive to levels of inorganic phosphate, the effect of phosphate in the growth media was examined. Varying the concentration from 2 mM to 2 μM had no effect on the specific activity of the enzyme in *P. vulgaris*. While the enzyme appears to be constitutive in the case of facultative anaerobes, the specific activity of the enzyme was generally lower when grown under anaerobic conditions.

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THE DESIGN AND IMPLEMENTATION OF A DOWN-LINE LOADER

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ABSTRACT

A system to load a PDP-8/E which acts as a concentrator for a DEC-10 computer system is discussed and developed. The PDP-8/E and DEC-10 are linked by a direct synchronous line over a distance of approximately 1/2 mi.

INTRODUCTION

Acting as a concentrator, the operating system of the PDP-8/E places 16 independent devices in contact with the DEC-10 over a single communications line. The operating system of the PDP-8/E periodically requires reloading due to system crashes or programming changes. Due to hardware constraints this operating system is currently being loaded by paper tape with an ASR-33 teletype. The only other input device available to the PDP-8/E is a card reader. Loading by cards has been performed in the past but is inconvenient because the installation does not have a card punch capability. Periodic software changes will not allow for a permanent card deck to be used. Acquisition of additional input devices for loading purposes is ruled out due to cost.

The alternative methods of loading involve down-line loading schemes which do not require additional hardware and which provide excellent performance.

THE DOWN-LINE LOADER

In general a loader is a program which accepts object code, places it in memory and initiates execution. The phrase "down-line loader" implies that a particular situation exists. First, that communication exists between a larger host processor and a smaller target processor which has been dedicated to a specific task or is being used in a distributed data processing network. Second, communication has been interrupted due to the destruction of the operating system within the target processor and that loading is to occur from the host processor to reestablish the operating system of the target processor and restore normal communications between the two.

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Down-line loading requires that the target processor first be loaded by some means to reestablish communications. This initial program loading or bootstrapping should enable the target processor to communicate with the host processor and receive and load object programs sent by the host processor. Once the target processor's operating system has been restored, normal operation can resume.

The down-line loading idea can also be applied in other situations where any independent target processor may not have a desirable program storage capability. In many instances processors are used to monitor or control mechanical devices under varying circumstances. In some cases the infrequent need for loading software cannot justify the cost of additional hardware. If communications could be established with a host processor a loading procedure could be developed which would allow the target processor to utilize the storage capability of the host and in many cases decrease the time required to access and load programs. Therefore, the target processor would not require any auxiliary storage capability other than that required for the initial program loader, which could take the form of a hardware bootstrap loader or a PROM. All other software such as assemblers, editors, or utilities could reside with the host and be loaded only as required.

TELEPHONE LINE LOADERS

This loading scheme involves communication between the 2 central processors over a telephone line at a rate of 300 baud. The PDP-8/E is connected to the DEC-10 via a telephone link so that the DEC-10 can output data to the PDP-8/E as if the PDP-8/E were a remote terminal programmed to receive, format, and load data as required.

Contact with the DEC-10 is established by dialing in on the telephone. Using the acoustic coupler and a terminal we login and reach monitor level to execute a program on the DEC-10 which is used to access disk files that contain PDP-8/E. object code and to output these files to the PDP-8/E. Upon execution, this program waits for an ACK (positive acknowledgement) character from the PDP-8/E before output of the desired object code begins. At this point the terminal is disconnected from the coupler in order to connect the PDP-8/E which is assumed to be in an unloaded state. Therefore, the loader which accepts object code from the DEC-10 must itself be loaded into memory. Fortunately, the PDP-8/E has an M18-E hardware bootstrap loader which has a capacity of 32 twelve bit words. The M18-E can load an initial memory address, deposit 32 words sequentially into memory, load a memory start address, and begin execution of the loaded program. Due to the size restriction placed on the loader by the M18-E, it is not possible to encode a 32 word loader capable of acknowledging the DEC-10 and receiving and loading binary-formatted object code with the attendant checksumming and handshaking. It is possible, however, to write a loader to send an ACK to the DEC-10 and receive and load RIM (read in mode) formatted object code without the checksumming and handshaking being done.

RIM format requires that each instruction in object code be preceded by the address at which the instruction is to be loaded. Binary format is similar to RIM

format except that only the initial address in a series of consecutive instructions is specified. The binary loader must then recognize new addresses or increment the current address when loading binary-formatted files. RIM format allows for a simpler loader because of the regular address/instruction order. However, these additional addresses increase load time considerably. It is desirable for the RIM format loader to load another larger loader capable of loading binary formatted files. This first RIM format loader is referred to as a TRIM (telephone read in mode) loader.

TRIM LOADER

The TRIM loader must satisfy the following requirements. First, it must send an ACK to the host to initialize output. As input is received from the host, the TRIM loader must distinguish between an address, an instruction to be loaded, or a leader-trailer, and must load each instruction at the proper address. Leader-trailer codes delimit the object code produced by the assembler and are discarded by the TRIM loader.

Each PDP-8/E address and instruction is 12 bits in length. Data sent over the telephone line by the DEC-10 is in groups of 8 bits requiring each PDP-8/E address or instruction to be sent in 2 parts. each containing 2 identification bits and 6 data bits. Leader-trailer requires only 6 bits and may be sent in 1 part.

The 2 identification bits are used only in the 1st half of the instruction or address that is sent. The first 2 bits of a required 2nd half are always off.

As each 1st half is received it is identified. If an address is identified, the 2nd half is received and the full address is formed and placed in a location reserved for the temporary storing of current addresses. If an instruction is received, the full instruction is formed after receiving the 2nd half and the instruction is loaded indirectly on the current address.

The following is a flowchart of the TRIM loader. Note that the algorithm has no logical end. All RIM object code that is loaded overlays the TRIM loader to cause a transfer to the start address of the loaded object code.

TRIM Flowchart

(T0) Send an ACK to the host

(T1) If a character has been received from the host THEN (T2) ELSE (T1)

(T2) Place the character in the AC (accumulator)

(T3) Place the character in the low half of the AC and the 1st ID bit in the LINK (rotate the AC left 5 bits)

(T4) IF bit 0 is set THEN (T5) ELSE (T10)

(T5) IF the link is set THEN (T13) ELSE (T6)

(T6) Execute change to data field 1

(T7) GO TO (T1)

(T10) IF LINK is set THEN (T11) ELSE (T13)

(T11) This character is a leader-trailer code so disregard

(T12) GO TO (T1)

(T13) Place the 2nd ID bit in the LINK (rotate AC left 1 bit)

(T14) If a character has been received from the host THEN (T15) ELSE (T14)

(T15) Place the character in the high half of the AC

(T16) IF LINK is set THEN (T17) ELSE (T21)

(T17) TEMP = AC

(T20) GO TO (T1)

(T21) LOC (TEMP) = AC

(T22) GO TO (T1)

THE BINARY LOADER

After the TRIM loader has been placed in the memory of the PDP-8/E by the M18-E the PDP-8/E is able to load the binary loader. The TRIM loader, although used here to load the binary loader, could be used to load any RIM formatted file without having to use the binary loader. Loading the binary loader in order to load a binary-formatted file may not be as fast as loading that same file in RIM format using the TRIM loader. A relatively short file could be loaded much faster in RIM format. The loading of a large file however, would be much slower using RIM format due to the time required to output and process the additional addresses. This time exceeds the time required to load the binary loader and then load the same file using binary format. Also the possibility of extraneous characters being placed in the transmission line, referred to as line noise, is increased as load time increases and could result in an improper load using the TRIM loader.

Earlier it was mentioned that the host processor would execute a program to output desired object code to the target processor. The TRIM loader by design will generally load the binary loader. The host program, referred to as RIMPRT must then access and output the binary loader in RIM format when an ACK is received from the TRIM loader. For convenience, all PDP-8/E object code is assumed to be in binary format which will require RIMPRT to change binary-formatted code to RIM format before output. This requirement is easily implemented and does not increase load time due to the slow transmission rate of the telephone line. TRIM and RIMPRT are always used together to load RIM formatted code.

RIMPRT Flowchart

(R0) Open channel for input
(R1) Open channel for output
(R2) Strip off leader-trailer codes from object code
(R3) Read next character
(R4) IF character is leader-trailer THEN (R22) ELSE (R5)
(R5) IF character is field change THEN (R6) ELSE (R10)
(R6) Output character to target
(R7) GO TO (R3)
(R10) IF character is an address THEN (R11) ELSE (R13)
(R11) Read second character and form current address
(R12) GO TO (R3)
(R13) Separate current address into 2 bytes
(R14) Output both address bytes
(R15) Output current character
(R16) Read 2nd half of the instruction
(R17) Output 2nd half
(R20) Increment current address
(R21) GO TO (R3)
(R22) END

The binary loader must be able to request and load any binary-formatted file that the host may have stored on disk. To enable the user to request files in a convenient manner, the binary loader includes a device driver to allow for user I/O with the host. The binary loader performs error checking and has the ability to request retransmission of data when errors are detected. If larger programs are to be loaded, the binary loader should be as small as possible.

Because of these constraints the binary loader, which is referred to as IMPORT, has 2 modes of operation: communications mode and load mode. Communications mode provides a device driver that allows the user to use a terminal device connected to the PDP-8/E to access the host via the telephone link. Any character typed on the user's console is accepted by IMPORT and sent to the host. The host receives the character and generates an echo of that character. IMPORT receives the character that has been echoed and sends it to the user's console. During communications mode the user has full use of the facilities of the host processor.

At some point the user can request that a binary file be loaded from the host. This is done by the execution of a host program similar to RIMPRT. This program, called EXPORT, can access binary-formatted object code and output it in that form. When the particular object code file has been determined, EXPORT sends a control character that IMPORT will recognize as the beginning of a load sequence and will enter load mode. When load mode has been initiated IMPORT will not output to the user's console, but will accept all data as object code to be loaded. Since error checking is to be done, EXPORT sends data in blocks containing a checksum and a word count in addition to the object code. Should an error in transmission occur, IMPORT requests EXPORT to retransmit the last block of data until it is received correctly or until a predetermined error count is exceeded. EXPORT sends data in the same manner as RIMPRT, 8 bits at a time. The first 2 bits are used for identification, the last 6 bits for data. Since object code is sent in blocks containing control information, IMPORT always considers the first 8 bits of each block to be a checksum, the second 8 bits to be a word count, and the remainder to be object code to be identified by their identification bits.

There may be cases that require object code to be loaded into memory where IMPORT is currently executing. A buffer area the size of a particularly vulnerable part of IMPORT has been set aside in the upper portion of the second 4K field. Code that is to be loaded in the same memory locations as IMPORT is placed in the corresponding relative locations in this temporary buffer.

At the conclusion of each load EXPORT sends another control character to terminate load mode, writes a load summary, and places the user terminal back into communication with the host. At this point the user may load additional object code, use the facilities of the host, or dump the temporary buffer overlaying IMPORT and begin execution of the loaded code. To dump the temporary buffer the user must type a control G, which IMPORT will recognize from communications mode to cause a transfer of control to a dump routine located near the buffer. This routine will dump the buffer and print a "load completed" message on the user's console.

The following are flowcharts of IMPORT and EXPORT.

IMPORT Flowchart

(I0) Initialize variables

(I1) IF an interrupt occurs THEN (I2) ELSE (I1)

(I2) IF an interrupt from the host to user device occurs THEN (I7) ELSE (I3)

(I3) IF an interrupt from the user's keyboard occurs THEN (I5) ELSE (I4)

(I4) IF an interrupt from the user's printer occurs THEN (I15) ELSE (I1)

(I5) Read character from user keyboard and send to the host

(I6) GO TO (I1)

(I7) Read character from the host
(I10) IF in load mode THEN (IL0) ELSE (I11)
(I11) If character is dump control character THEN (ID0) ELSE (I12)
(I12) IF load mode control character THEN (I13) ELSE (I15)
(I13) Set load mode
(I14) GO TO (I1)
(I15) Write character to buffer
(I16) IF output to user console is in progress THEN (I1) ELSE (I5)
(I17) Output a character from the buffer to the user printer
(I20) GO TO (I1)
(IL0) IF byte flag set THEN (IL26) ELSE (IL1)
(IL1) IF word count has been received then (IL6) ELSE (IL2)
(IL2) IF checksum has been received THEN (IL5) ELSE (IL3)
(IL3) Initialize checksum
(IL4) GO TO (I1)
(IL5) Initialize word count
(IL6) GO TO (I1)
(IL7) IF the character is the 1st half of an instruction THEN (IL10) ELSE (IL14)
(IL10) Move the character to low half of AC and store
(IL11) Increase checksum
(IL12) Set byte flag
(IL13) GO TO (I1)
(IL14) IF the character is the 1st half of an address THEN (IL15) ELSE (IL17)
(IL15) Set address flag
(IL16) GO TO (IL10)
(IL17) IF the character is a field change THEN (IL20) ELSE (IL21)
(IL20) Set new data field
(IL20) GO TO (IL45)
(IL21) IF the character is a terminate load code THEN (IL23) ELSE (IL25)
(IL23) Clear load mode
(IL24) GO TO (I1)
(IL25) The character is trash so ignore
(IL26) GO TO (I1)

(IL27) Turn off byte flag
(IL30) Increase checksum
(IL31) Join bytes to form word
(IL32) IF address flag set THEN (IL33) ELSE (IL36)
(IL33) Clear address flag
(IL34) Set CA (current address)
(IL35) GO TO (IL45)
(IL36) IF this data should be buffered THEN (IL37) ELSE (IL41)
(IL37) Store the word in the buffer
(IL40) GO TO (IL44)
(IL41) Select the current data field
(IL42) LOC (CA) = WORD
(IL43) Restore original data field
(IL44) Increment current address
(IL45) Increment word count
(IL46) IF this is the last word THEN (IL47) ELSE (I1)
(IL47) IF checksum is correct THEN (IL50) ELSE (IL52)
(IL50) Send an ACK to the host
(IL51) GO TO (I1)
(IL52) Send retransmit to the host
(IL53) Clear line
(IL54) IF line is clear THEN (I1) ELSE (IL53)
(ID0) Dump the buffer
(ID1) Print "LOAD COMPLETE"
(ID2) GO TO the initial starting location of the loaded object code.

EXPORT Flowchart

(E0) Open channel for input
(E1) Open channel for output
(E2) Strip off leader-trailer codes from object code
(E3) Read next character and store
(E4) Read 2nd half of address and store
(E5) Initialize checksum and word count
(E6) Read next character
(E7) IF character is a leader-trailer THEN (E30) ELSE (E10)
(E10) IF character is an address THEN (E11) ELSE (E16)

(E11) Send current buffer
(E12) IF reply from target is an ACK THEN (E13) ELSE (E11)
(E13) Store address half
(E14) Read 2nd half of address, form full address, and store
(E15) Initialize checksum and word count
(E16) Strip off consecutive addresses and re-initialize if necessary
(E17) GO TO (E6)
(E20) IF word count is equal to the buffer size THEN (E21) ELSE (E25)
(E21) Send the current buffer
(E22) IF reply from target is ACK THEN (E23) ELSE (E21)
(E23) Store current address
(E24) Re-initialize checksum and word count
(E25) Store character in buffer
(E26) Increment word count
(E27) GO TO (E6)
(E30) Output current buffer
(E31) Terminate load mode
(E32) Output load summary

CONCLUSIONS

In summary, the design and implementation of a down-line loading system have been discussed. The need for this system came from the requirement of a target processor to be loaded by a more efficient means, without acquiring additional hardware. The existing hardware environment provided communication facilities to a host processor and therefore the basic elements existed for the development of a down-line loader.

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A COMPARISON OF SORTING ALGORITHMS

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ABSTRACT

Thirteen sorting algorithms described by Donovan (1972) and Knuth (1973) are classified as to type, data structure, advantages and disadvantages. Comparative computer sorting times for each algorithm are obtained for various file sizes, data structures, and degree of initial order.

INTRODUCTION

A PDP 11/20 with 16K words of primary memory was used as the host computer for this study. Each algorithm was used to sort 3 sets of records (250, 500, 750) whose keys are randomly generated integers in the interval (0, 10000). Each record consists of a key and 4 words of satellite information associated with that key. Satellite information is any information that is integral to a record but which does not affect the ordering of the records. For example, a record may contain a name, a social security number and a mailing address, the sorting being applied to only one of these items. Satellite information was used in this study because some of the sorting algorithms use a linked data structure. The advantages of these algorithms are not seen if files consisting only of keys are used because their increase in speed is due to eliminating the moving of long records from one memory area to another. Inclusion of satellite information improves such studies as that of Lorin (1975) which do not include such information. Such algorithms as the bubble sort applied to files whose records contain 1 word of satellite information require almost twice the time necessary to sort keys only. The radix list sort, however, is unaffected by the amount of satellite information present because only links are manipulated. Therefore, this aspect of file structure is an important consideration in the selection of an appropriate sorting algorithm. Best case and worst case comparisons were obtained by applying each algorithm to the same records which had been arranged in sorted order (best case) and then to the same records which had been arranged in reverse order (worst case).

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DEFINITIONS

The file to be sorted consists of N records denoted $R_1, R_2, \ldots, R_N$. Associated with each record R_i is a key denoted K_i. The file is said to be sorted if $K_1 \leq K_2 \leq \ldots \leq K_N$ where the relation '$\leq$' denotes a defined order relation which may be numeric, alphabetic, or any other desired ordering or collating sequence. Each algorithm was constructed as an internal sort; that is, all records are stored and sorted in primary memory. The memory requirement for each algorithm is determined primarily by the size of each record and by whether or not the algorithm performs an in-place sort. A file is said to be sorted in-place if the sorted file occupies the same memory locations that the unsorted file occupies; that is, the entire file never appears more than once in memory.

COMPARISONS AND MOVES

Each sorting technique has strengths and weaknesses which indicate its applicability to particular data structures.

Table 1 shows the time required for each algorithm to sort each file. Random refers to the original, randomly ordered file. Reverse refers to the sorted file arranged in reverse order.

The primary considerations involved in selecting an internal sorting algorithm are (1) number of comparisons of keys, (2) number of moves of records, (3) amount of memory required, and (4) file data structure.

Counting algorithms are interesting in their simplicity but they are not particularly efficient because they require a comparison of all distinct pairs of keys (K_i, K_j). They also require additional memory because they are not in-place sorts.

Comparison counting utilizes the fact that, in the final sequence of records, the j-th key is greater than exactly j - 1 of the other keys. Each key is compared to all other keys, the results of these comparisons being used to determine the final location of each record in the output area. However, an output area equal to the file size and a count area of length N are also required. No significant changes in sorting time are observed for best and worst case situations.

Insertion algorithms insert record R_i into a previously sorted file of records $R_1, R_2, \ldots, R_{i-1}$, $i = 2, 3, \ldots, N$. These algorithms generally require fewer comparisons of keys; particularly if, as in the diminishing increment sort, the comparisons are selected intelligently. The diminishing increment sort achieves time reduction through movement of records relatively long distances in memory to reduce the total number of moves required. List insertion decreases sorting time by making use of a linked data structure (manipulating links rather than keys) to eliminate moving records around in memory.

Straight insertion is asymptotically less efficient than diminishing increment because records are moved to adjacent locations, although smaller files are handled more efficiently with straight insertion. Address calculation, finally, improves on

TABLE 1
Sorting Times (1/60 sec)

Algorithm	N=250 Random	N=250 Sorted	N=250 Reverse	N=500 Random	N=500 Sorted	N=500 Reverse	N=750 Random	N=750 Sorted	N=750 Reverse
Counting									
Comparison	238	204	270	933	796	1065	2086	1780	2385
Insertion									
List	120	3	231	470	6	921	1051	9	2070
Diminishing Increment	63	28	48	150	61	117	253	94	184
*Straight	54	2	104	211	3	413	471	4	928
Address Calculation	22	11	33	69	21	113	142	31	239
Exchange									
Bubble	842	2	1465	3383	4	5871	7608	5	13216
*Batcher's Parallel	32	25	28	83	61	69	142	107	117
*Quicksort	19	124	150	45	476	562	74	1048	1227
Selection									
Straight	154	145	284	583	561	1117	1289	1253	2491
Heap	56	60	51	126	134	117	203	214	189
Merge									
Natural Two-way	51	13	13	127	26	26	190	39	38
List	29	23	23	66	51	52	107	83	81
Distribution									
Radix List	28	28	28	56	56	55	83	83	83

*Implemented in MACRO-11 Assembly Language. All others implemented in FORTRAN.

list insertion by linking records into several subfiles having similar keys and then by linking these subfiles.

Exchange sorting algorithms swap records that are out of order until the file is in correct order. Time requirements are reduced by intelligent selection of keys to compare (Batcher's parallel algorithm) although some (Quicksort) require modest amounts of additional memory to store extra bookkeeping information. The bubble sort is least efficient because adjacent records are swapped in memory if they are out of order thus increasing significantly the time requirement for moves, especially if the record size is very large. However, if the file is ordered, or almost so, to begin with, the bubble sort executes very quickly.

Selection sorting algorithms select records with the smallest key and place them in their final position in the sorted file by exchanging them with the records occupying those positions. Heap sort is the most efficient selection sort because it utilizes a tree structure to simplify the selection process. Only the worst case time for straight selection is significantly different because the maximum number of comparisons must be made in the selection process.

Merge sorts in general combine 2 or more sorted subfiles into 1 sorted file. However, this idea can be adapted to sorting randomly ordered files by merging longer and longer runs of records where a run is defined as 1 or more records which are already arranged in proper order. The natural 2-way merge requires an additional N memory locations for a file output area. This disadvantage is overcome by the list merge which only requires an additional field in each record for a link pointer to the next record. Merge sorts show significant time improvements for the best and worst cases because the naturally occuring runs are quite lengthy.

Distribution sorts divide the file to be sorted into 2 or more subfiles whose keys have some characteristic in common; for example, ones digit of keys are equal. These subfiles form a new file which is again divided into several subfiles based on another characteristic of keys; for example, tens digit of keys are equal. This process is repeated until the file is sorted. The speed of the radix list sort is achieved at the expense of a link field in each record as well as 2M additional link pointers where M is the radix utilized in the sort; for example, a base 8, 10, or 16 representation of the keys.

MEMORY REQUIREMENTS

Table 2 lists the amount of primary memory required for record storage, link field storage, and auxiliary table storage. In the column for record storage, N means the records appear once in memory; 2N means the records appear twice in storage. In this study, 5 words are required for each record; 1 for the key and 4 for the satellite information. Link fields and auxiliary tables require 1 word/item.

MACHINE AND LANGUAGE DEPENDENCE

In specific instances, the host computer and host language have a certain effect upon execution time. The PDP-11 with a moderately fast instruction time of

TABLE 2
Memory Requirements

Algorithm	Records	Links	Auxiliary
Counting			
Comparison	2N		N
Insertion			
List	N	N	
Diminishing Increment	N		T(small)
Straight	N		
Address Calculation	N	N	
Exchange			
Bubble	N		
Batcher's Parallel	N		
Quicksort	N		P(small)
Selection			
Straight	N		
Heap	N		
Merge			
Natural 2-way	2N		
List	N	N	
Distribution			
Radix List	N	N	

approximately 2-2.5 μsec is an appropriate machine for computer sorting. However, the effect of machine on execution is essentially linear because the average number of instructions executed changes little from machine to machine.

The same comments can be made concerning the host languages of FORTRAN and MACRO-11 used in this study. While FORTRAN generally produces less efficient code than an able assembly programmer, the effect on execution time is also essentially linear.

CONCLUSIONS

Overall the radix list sort seems to be the best internal sorting technique for large records even though it does not achieve the increased best case speeds attained by other sorts such as the bubble, list insertion or natural 2-way merge. Batcher's parallel algorithm seems to be the best if record size is not too large even though best case speeds are not dramatically faster. However, Batcher's parallel algorithm does allow many computations to be performed in parallel so that its implementation on a computer with parallel processing capabilities would evidence even faster sorting times than given here.

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HASH CODES FOR NON-PRIME TABLE SIZES

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ABSTRACT

A set of conditions is given which ensure that members of a family of hash codes achieve full period. Several members of the family which have been reported in the literature are discussed. A linear and quadratic hash code which can be applied to non-prime table sizes are presented and examined. The results of a series of simulation experiments designed to examine the performance of these methods are presented and examined. These results show both hash codes in close agreement with theoretical performance of hash codes which are not affected by primary and secondary clustering.

INTRODUCTION

Hash coding is the name of a group of techniques for organizing a file or table of data in such a way that the items can be retrieved very rapidly. A good description of hash methods is provided by Maurer and Lewis (1975). Specifically a hash code is an algorithm that will produce a sequence of possible table addresses based on the value of the search key of an entry. This list of addresses is known as the hash sequence of the entry. Entries are inserted in the table at the first address of the hash sequence that is unoccupied by another entry. Since the hash sequencies of different items are generally different, entries can usually be located near the beginning of their hash sequences. The first member of a hash sequence, h_0, is known as the initial probe address. The initial probe calculation is usually a function of the search key. Subsequent members of the sequence are denoted by h_i for $i > 0$ where i is known as the hash index. Each h_i is computed as a function of the initial probe address and possibly the search key and the hash index. If the hash sequence contains each address exactly once before repeating, it is said to have full period. Full period hash sequences are needed so that items can always be inserted if a table is not completely full.

Efficient hash codes such as the linear quotient hash code (Bell and Kaman, 1970) and the quadratic quotient hash code (Bell, 1970) are restricted to tables with sizes that are prime numbers. This restriction can result in inefficient implementations when space allocations for the table (or file) are made in units which do not match the table size. Computational efficiency could sometimes

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be improved if the table size could be chosen to take advantage of machine features. This paper defines a family of hash codes many of which can be applied to non-prime tables. The performance of 2 members of the family is examined.

FULL TABLE SEARCHING

Ackerman (1974) pointed out the quadratic search hash code could be applied to tables of size p^n for any prime p. This is accomplished by computing the hash sequence for a numeric representation of a search key, K, as

$$h_i(K) = [h_0(K) + ai + bi^2] \bmod p^n$$

where p divides b but not a. Under these conditions the hash sequence is exhaustive; each location is examined exactly once before the sequence repeats. Batagelj (1975) showed the quadratic search hash code could be applied with other non-prime table sizes. The following theorem establishes a similar result for a broader class of table sizes.

Theorem: The hash sequence $h_i(K) = [h_0 + ai + bi^2] \bmod n$ has full period, n, if $(a,n) = 1$ and if $p \mid b$ for all prime factors p of n.

Proof: Assume $h_i = h_k$ for $i < k$
Let $j = k - i$
$h_0 + ai + bi^2 \equiv h_0 + a(i+j) + b(i+j)^2 \bmod n$
$h_0 + ai + bi^2 \equiv h_0 + ai + aj + bi^2 + 2bij + bj^2 \bmod n$
$j(a + 2bi + bj) \equiv 0 \bmod n$
If p is any prime factor of n, then $p \mid 2bi$ and $p \mid bj$ and, since p does not divide a, $(a + 2bj + bj, n) = 1$.
$j(a + 2bi + bj)/n = k$ for some positive integer k. Since n and $(a + 2bi + bj)$ are relatively prime, j must be a multiple of n. Therefore, the hash sequence has a full period of n.

Under the conditions of the theorem a family of hash codes is defined. Ackerman's (1974) quadratic search hash code is a member of that family. Luccio (1972) suggested a method known as the weighted increment linear search with the following hash sequence:

$$h_i(K) = [h_0(K) + (2h_0(K) + 1)i] \bmod 2^r.$$

Since $2h_0(K) + 1$ is an odd number, $(2h_0(K) + 1, 2^r) = 1$. Therefore, with $b = 0$, Luccio's (1972) method is a member of the family defined by the theorem.

If $a = 1$ and $b = 0$, the sequence produced is identically that of the linear search hash code. This hash code exhibits poor performance because it is subject to primary and secondary clustering. Bell (1970) defines both types of clustering and gives an excellent discussion of its effects. With respect to Bell's (1970)

work, note that whenever the table size, n, is a prime or the product of distinct prime factors or when $b = 0$, the sequence produced will be linear with respect to the hash index. This means that primary clustering will not be completely eliminated.

If n is prime, the quadratic term vanishes, but a can be chosen as any arbitrary value other than 0 or a multiple of n. In particular, if a is chosen to be the integer quotient of the key divided by the table size (1 if the quotient is congruent to 0 mod n), the hash sequence is identically that of the linear quotient hash code. Bell and Kaman (1970) have shown that while neither primary or secondary clustering are completely eliminated, they are very effectively controlled by the linear quotient method. Hash sequences of distinct entries are in general different because their quotients are, in nearly all cases, different.

NON-PRIME LINEAR AND QUADRATIC HASH CODES

In addition to these hash codes, the family contains others that can be applied to non-prime table sizes. If n is non-prime the constant b can be chosen as 0 or as the product of the distinct prime factors of n (denoted by p^*). For example: $n = 128$, $p^* = 2$; $n = 500$, $p^* = 10$; $n = 1024$, $p^* = 2$; $n = 7000$, $p^* = 70$; etc. The value of a must be chosen relatively prime to n. One possibility is to choose a as $p^* \cdot g(K) + 1$, where $g(K)$ is an arbitrary integer function of the key, K.

The linear quotient hash code and Bell's (1970) quadratic quotient hash code suggest 2 methods for non-prime table sizes. First, if $b = 0$ and $a = p^* \cdot Q(K) + 1$ where $Q(K) = \lfloor K/n \rfloor$, the resulting hash sequence is given by

$$h_i(K) = [h_0(K) + (p^* \cdot Q(K) + 1) \cdot i] \bmod n.$$

This method will be referred to as the non-prime linear quotient hash code (NLQH). As with the Bell and Kaman's (1970) linear quotient method, the non-prime linear quotient hash code does not completely eliminate clustering. However, since the hash sequences are functions of different keys clustering is controlled.

If a is chosen as before and if b is the product of distinct prime factors of n, a method referred to as the non-prime quadratic quotient hash code (NQQH) is given by the following hash sequence:

$$h_i(K) = [h_0(K) + (p^* \cdot Q(K) + 1) \cdot i + p^* \cdot i^2] \bmod n.$$

This hash sequence is quadratic in the hash sequence index. Therefore, as pointed out by Bell (1970) primary clustering is eliminated. Secondary clustering, though not completely eliminated, will be controlled since the sequence is based on the key.

HASH CODE PERFORMANCE

The performance of the methods described in the previous section is examined by reviewing the results of a series of simulation experiments. Both hash codes

were applied to 12 tables with sizes from 128 to 10,000 locations. Each of the tables was filled to a specified fraction with uniformly distributed random keys. For each fill factor, the average number of probes needed to retrieve an entry was computed. The average number of probes needed to insert an entry was estimated by attempting to insert 500 random keys. Assuming there is no clustering, the theoretical values are given by

$$R = -\alpha^{-1} \ln(1 - \alpha)$$

and $$I = (1 - \alpha) - 1 \qquad (0 < \alpha < 1)$$

where R and I are the average number of probes per retrieval and insertion respectively, and where α is the fill factor (the ratio of filled locations and the table size). The values for each of the 12 table sizes are averaged together. Table 1 contains the theoretical and observed values for both methods.

TABLE 1

Results of Simulation Experiments

	Retrieval			Insertion		
α	R	NLQH[1]	NQQH[1]	I	NLQH[1]	NQQH[1]
0.50	1.386	1.38	1.38	2.000	2.02	2.00
0.75	1.848	1.87	1.85	4.000	3.98	3.95
0.80	2.012	2.04	2.03	5.000	5.06	5.01
0.85	2.232	2.25	2.25	6.667	6.64	6.59
0.90	2.558	2.56	2.54	10.000	9.98	10.10
0.95	3.153	3.14	3.07	20.000	19.09	19.26
0.99	4.652	4.32	4.27	100.000	80.62	81.66

[1] NLQH and NQQH stand for the non-prime linear and quadratic quotient methods.

The results indicate that both methods are not appreciably affected by clustering. The results for retrieval show that the non-prime quadratic quotient method performed slightly better for larger fill factors. However, the non-prime linear quotient hash sequence is easier and faster to compute. Since the quadratic method eliminates primary clustering and the linear method only attempts to control it, the suspicion exists that the difference may not be sampling error. To test the hypothesis that the differences are significant, a test of paired samples is based on a procedure described by Snedecor and Cochran (1972).

The average number of probes per retrieval was measured for the 12 different tables. The table size serves as a basis for pairing of the values. For each fill factor the following procedure is used:

The average deviation is computed as

$$\bar{D} = \frac{1}{12} \sum_{i=1}^{12} D_i$$

where $\bar{D}$ is the average deviation and D_i is the difference of the observed values (quadratic minus linear) for each table.

An estimate of the standard deviation of the differences is obtained by computing

$$s_D = \frac{\sum_{i=1}^{12} (D_i - \bar{D})^2}{11}$$

Thus $s_{\bar{D}} = s_D/12$ is an estimate of $\sigma_{\bar{D}}$. Finally the value of t is computed as

$$t = \frac{\bar{D} - \mu_D}{s_{\bar{D}}}$$

The statistic computed follows the Student's t-distribution with 11 degrees of freedom. Similar statistics are computed for the average insertion values.

The values of t are used to test the null hypotheses that $\mu_D = 0$ versus the alternative that $\mu_D > 0$. Table 2 contains the values of t for retrieval and insertion for several fill factors between 0.5 and 0.99. The 5% level of t in a 1-tailed test is 1.796 for 11 degrees of freedom. If the null hypothesis ($\mu_D = 0$) is true, the probability that $t \geq 1.796$ is 0.05. None of the hypotheses can be rejected at the 5% level on the basis of the collected data for these hash codes. The evidence does not indicate a significantly better performance of the non-prime quadratic quotient code in comparison to the non-prime linear quotient method.

TABLE 2
Computed Values of the Statistic t for Comparison of the Non-prime Linear Quadratic Hash Codes

α	Retrieval t	Insertion t
0.50	-0.80	1.44
0.75	0.64	0.54
0.80	0.44	0.76
0.85	-0.03	0.43
0.90	0.38	-0.57
0.95	0.87	-0.56
0.99	0.93	-0.49

INITIAL PROBE CALCULATION FOR NON-PRIME TABLES

Care should be taken when choosing methods for computing initial probe locations for non-prime hash tables. Lum, *et al.*, (1971) indicated that for many cases the remainder of the key divided by a divisor, D, gives good results. The divisor is usually taken as the table size. Lum's, *et al.*, (1971) results indicate that if a large number of keys are congruent modulo d and if D is a multiple of d, then poor performance can result. For example, if the table size is chosen as a power of 10 and if the keys are decimal integer identification numbers, the table

size would not be a good divisor. Lum's, *et al.*, (1971) study indicated that a prime divisor worked best but that odd divisors were almost as good. It was suggested that divisors be chosen that do not contain small prime factors. If the division method is to be used at all for non-prime tables, the divisor should not be the table size.

CONCLUSION

A technique for applying hash coding to non-prime table sizes has been presented. Conditions are established that insure that members of a family of hash codes attain full period hash sequences. Two members of this family, the non-prime linear and quadratic quotient hash codes, are examined. The results of a series of simulations indicate that these hash codes effectively control clustering. The results also show that there was no significant difference in the performance of the 2 methods even though one eliminates primary clustering while the other does not. The non-prime linear quotient hash code, which is relatively simple, does effectively control clustering.

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INVERTEBRATE MACRO-FOSSILS OF ERATH COUNTY, TEXAS

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ABSTRACT

Erath County, Texas, an area of approximately 1,085 mi^2, has rocks of Pennsylvanian and Cretaceous ages exposed. During Pennsylvanian time repeated transgressions and regressions of marine water are evident. The marine units are fossiliferous and where exposed reveal a diverse fauna. The fossil bearing Pennsylvanian units are limited in exposure to the northern part of the county.

A record of the transgression of the Cretaceous sea is found in the highly fossiliferous units exposed throughout the rest of the county. Cretaceous collecting sites are most numerous as reflected by the number of collecting sites reported. Within the 2 geologic periods, 84 species of invertebrates have been collected and shown by Plates 1 through 20.

INTRODUCTION

The purpose of this study is to place under a single cover the macro-fossils found in Erath County, Texas. Within the borders of Erath County, rock units belonging to the Pennsylvanian and Cretaceous periods are exposed. From collecting sites throughout the county 84 species were found. Fossiliferous Pennsylvanian outcrops are fewer than that of the Cretaceous, reflecting the relative extent of outcrop of the respective units within the county.

LOCATION AND EXTENT OF THE AREA

The area of study includes all of Erath County, Texas, which comprises an area of 1,085 mi^2 of North Central Texas. Erath County includes parts of 3 major physiographic provinces, the Grand Prairie, the West Cross Timbers and the North Central Prairie. Stephenville, the county seat, is centrally located in the county and is 65 mi southwest of Fort Worth on Highway 377.

PURPOSE AND SCOPE

The purpose of this study is to prepare under a single cover an inventory of as many species of invertebrate macro-fossils as the author could find during a 6-wk

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period of intensive collecting in the summer of 1973. Approximately 1200 mi travel was completed throughout the county to locate as many diverse species and collecting sites as possible. The author having collected in this area for a number of years realizes that new species are yet to be found. Over the years upon repeated field trips to a collecting site new species are found.

GEOLOGIC SETTING

Within the boundaries of Erath County there are exposed rocks of 2 great geologic periods. In the northern portion of the county, rocks of Pennsylvanian age are exposed. The Pennsylvanian period began approximately 310 million years ago and terminated some 275 million years ago. Rocks of Cretaceous age are exposed in the remainder of the county. The Cretaceous period began approximately 135 million years before the present and terminated about 65 million years ago.

During Pennsylvanian time the present day area of Erath County was a part of a low coastal plain and delta complex. Streams originating in adjacent upland areas transported a tremendous amount of sand and mud into this area. From time to time marine water transgressed across the region. These transgressions are marked by the occurrence of fossils of the then existing marine life. Since there was cyclic transgression and regression of the marine waters across the area, good fossil collecting sites are somewhat limited. Only in certain rock layers are fossils found and these exposures are widely scattered. Three Pennsylvanian collecting sites were located in the northern portion of the county. An excellent site is located on the Lowe Ranch west of Patillo, Texas. According to the recently published Geologic Atlas of Texas, Abilene Sheet, 1972, the fossils are within the Grindstone Creek Formation. This formation consists of 2 members, the lower unit is the Brannon Bridge Limestone and the upper unit is the Buck Creek Sandstone. Another collecting site, also within the Grindstone Creek Formation is located on the York Farm east of Patillo, Texas. Some overlap in species between the 2 aforementioned sites occur, but generally the yield is quite different. A 3rd Pennsylvanian site is located on the Thurber Ranch, northwest of Hannibal, Texas. Rocks exposed in this area are part of the Mingus formation. This formation consists of 3 members, the lower unit being the Santo Limestone, middle member the Dobbs Valley Sandstone and the upper unit the Goen Limestone.

During Cretaceous time the area of Erath County was once again inundated by marine water. The fossils associated with these marine deposits of the Cretaceous sea are common to those of shallow shelf deposition. Seven formations of Lower Cretaceous age are exposed in Erath County. In sequence oldest to the youngest, they are the Twin Mountains, Glen Rose, Paluxy, Walnut, Comanche Peak, Edwards and Kiamichi formations. The units yielding the most diverse fauna are the Walnut and Comanche Peak formations. Excellent exposures of the Walnut formation are found to the west, southwest and southeast of Stephenville. Comanche Peak exposures are limited to the area east and southeast of Stephenville. This unit

also yields a variety of fossils. Although the Walnut and Comanche Peak yield the largest number of species, the Glen Rose and Edwards formations contribute several species not found in other units.

COLLECTING LOCALITIES AND PLATES

Twenty collecting sites are shown below. These sites are located in all quadrants of the county to give maximum representation of the fauna present. Cretaceous sites are most numerous, with only 3 Pennsylvanian sites represented. This reflects the relative size of outcrop between the Cretaceous and Pennsylvanian in the county. Plate 21 shows an outcrop of the Walnut formation of Cretaceous age at collecting site No. 1. Plate 2 shows a view of the Grindstone Creek exposure on the York farm, collecting site No. 14. Plates 1 through 20 show the 84 species collected.

Collecting Localities

Locality 1	On Highway 67, 15 mi southeast of Stephenville from the intersection of Highways 281 and 67.
Locality 2	On FM 220, 5 mi southwest of Highways 67 and 220 intersection.
Locality 3	William Anderson farm, 3 mi south-southwest of Chalk Mountain, Texas.
Locality 4	On Highway 67, 12 mi southeast of Stephenville from the intersection of Highways 281 and 67
Locality 5	On FM 914, 2.5 mi southwest of Alexander, Texas.
Locality 6	On FM 219, 6 mi south of Purves Community.
Locality 7	On Highway 6, 1 mi northwest of Alexander, Texas.
Locality 8	On FM 220, 2.5 mi southeast of Duffau, Texas.
Locality 9	On FM 219, 1.5 mi north of Lingleville, Texas.
Locality 10	On FM 2303, 7 mi northwest of the intersection of FM 2303 and 108.
Locality 11	On FM 2156, 1 mi west of FM 219 and 2156 intersection.
Locality 12	On FM 219, 3 mi north of Bunyan, Texas.
Locality 13	Thurber Ranch.
Locality 14	Wayne York farm, 1 mi east of Patillo, Texas
Locality 15	T. T. Lowe Ranch, 4 mi west of Patillo, Texas.
Locality 16	On FM 3025, 2 mi east of the intersection of FM 108 and 3025.
Locality 17	On FM 1715, 1 mi northwest of Morgan Mill, Texas.
Locality 18	On FM 2157, 1 mi east of Cedar Point.
Locality 19	On Highway 377, 5 mi northeast of Stephenville.
Locality 20	On Highway 281, 2 mi north of the intersection of FM 8 and 281.

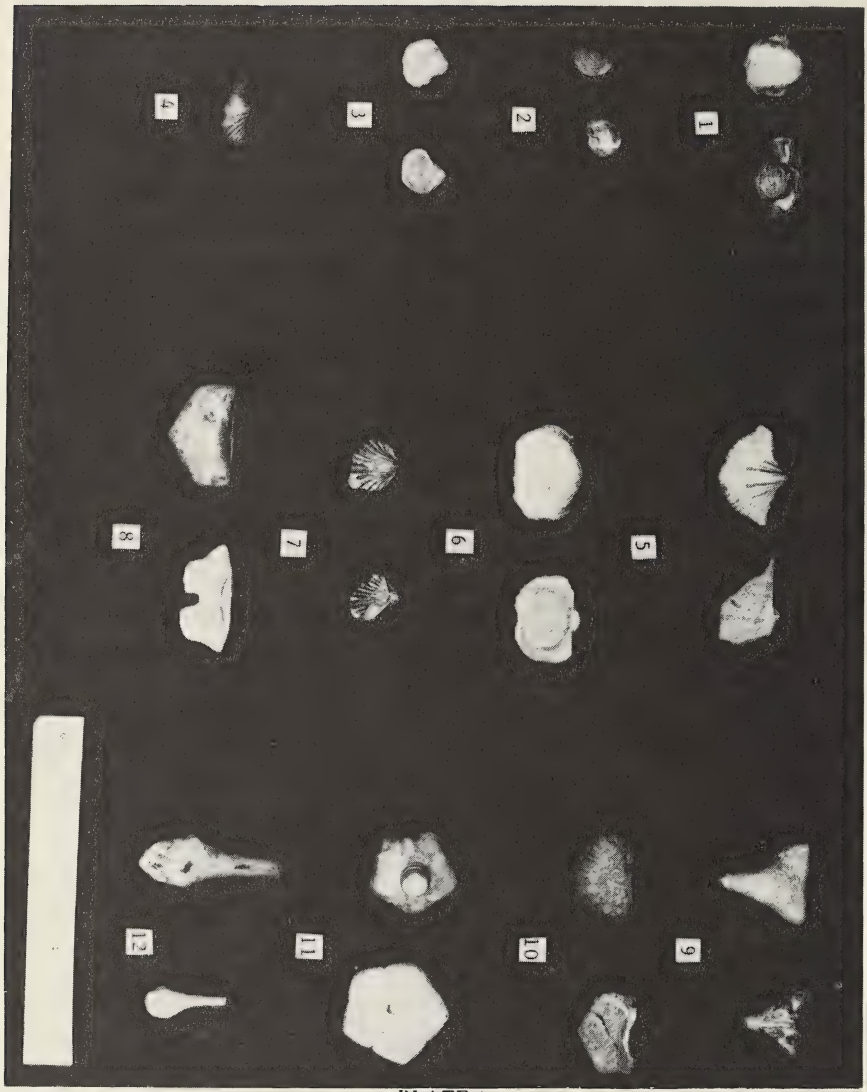

PLATE 1

FAUNA	LOCALITIES 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Pharkidonotus percarinatus*													X	X	X					
2. *Phanerotrema grayvillense*													X	X	X					
3. *Crurithyris planoconvexa*														X						
4. *Punctospirifer kentuckiensis*													X	X	X					
5. *Spiriferina kentuckiensis*													X	X	X					
6. *Marginifera muricatina*													X	X	X					
7. *Hustedia mormoni*														X						
8. *Delocrinus sp.**													X	X	X					
9. *Brachial acutus**													X	X	X					
10. *Facetal tuberculatus**													X	X						
11. *Apical regularis**													X	X						
12. *Hydreionocrinus**													X	X	X					

*Fragmentary remains of Crinoids

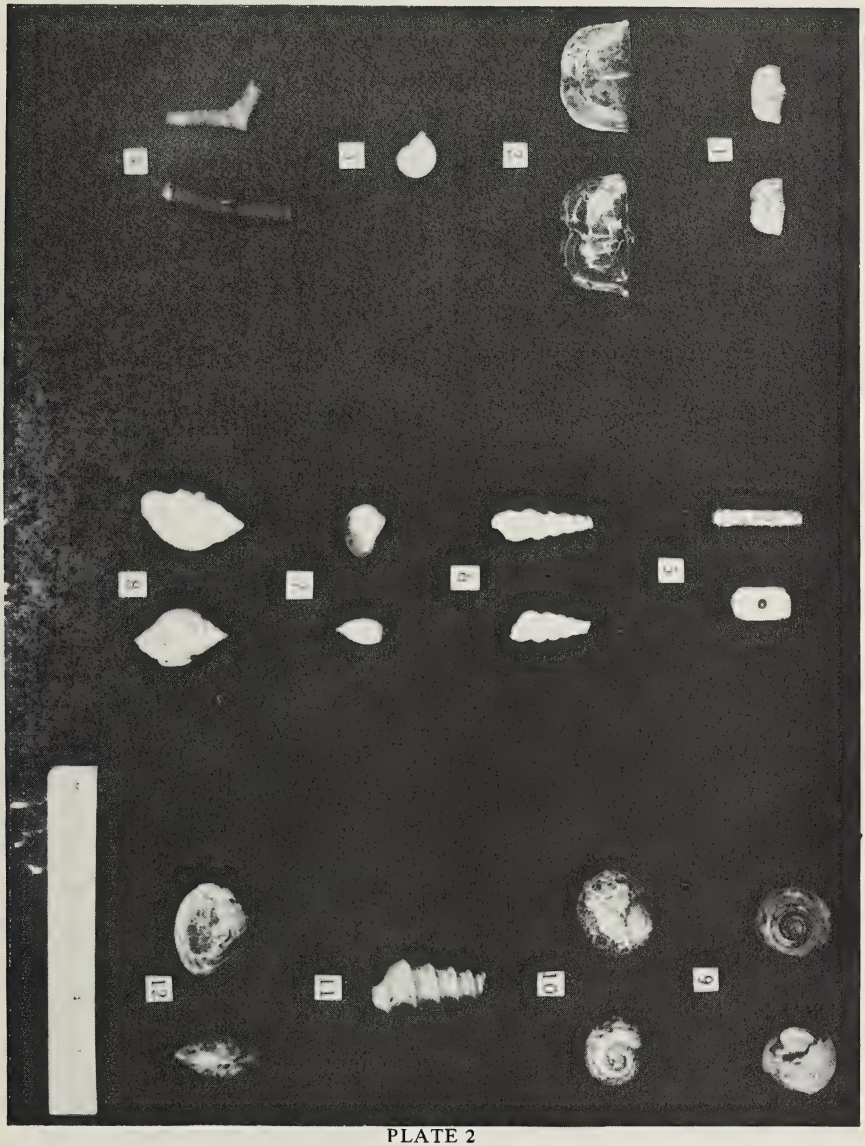

PLATE 2

FAUNA	LOCALITIES 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1. *Mesolobus mesolobus*	X X X
2. *Chonetina flemingi*	X X X
3. *Straparolus sp.*	X
4. *Rhombopora lepidodendroides*	X
5. *Echinocrinus* (Plate & Spine)	X X
6. *Pseudozygopluera sp.*	X
7. *Nucula anodontoides*	X X
8. *Sphaerodoma primigenia*	X X
9. *Worthenia tabulata*	X X X
10. *Trachydomia wheeleri*	X X
11. *Goniasma lasallensis*	X
12. *Nucula croneisi*	X

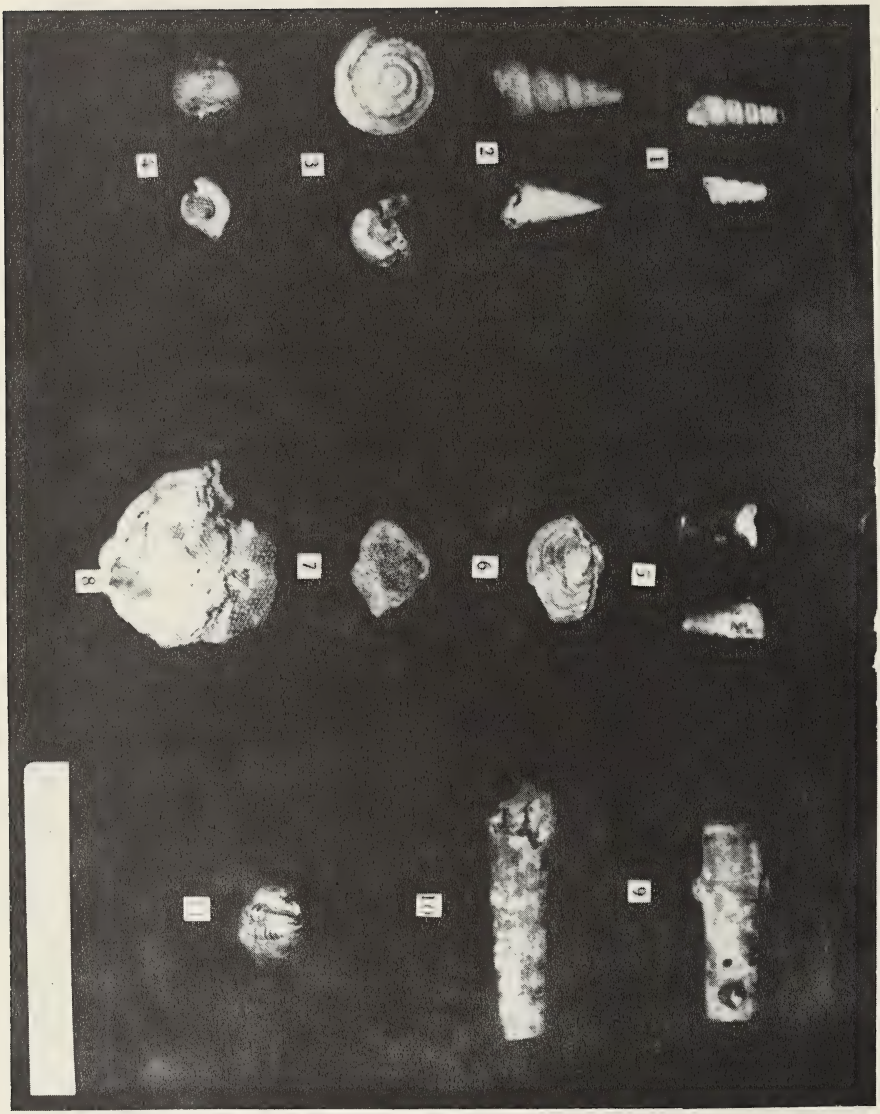

PLATE 3

FAUNA	LOCALITIES 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1. *Murchisonia sp.*	X
2. *Donaldina robusta*	X
3. *Trepospira depressa*	X
4. *Euphemus vittatus*	X X X
5. *Mooreoceras normale*	X X X
6. *Astartella sp.*	X X X
7. *Tabulipora sp.*	X X X
8. *Polypora spinulifera*	X
9. *Crinoid columnal*	X X X
10. *Dentalium sp.*	X
11. *Griffithides sp.*	X

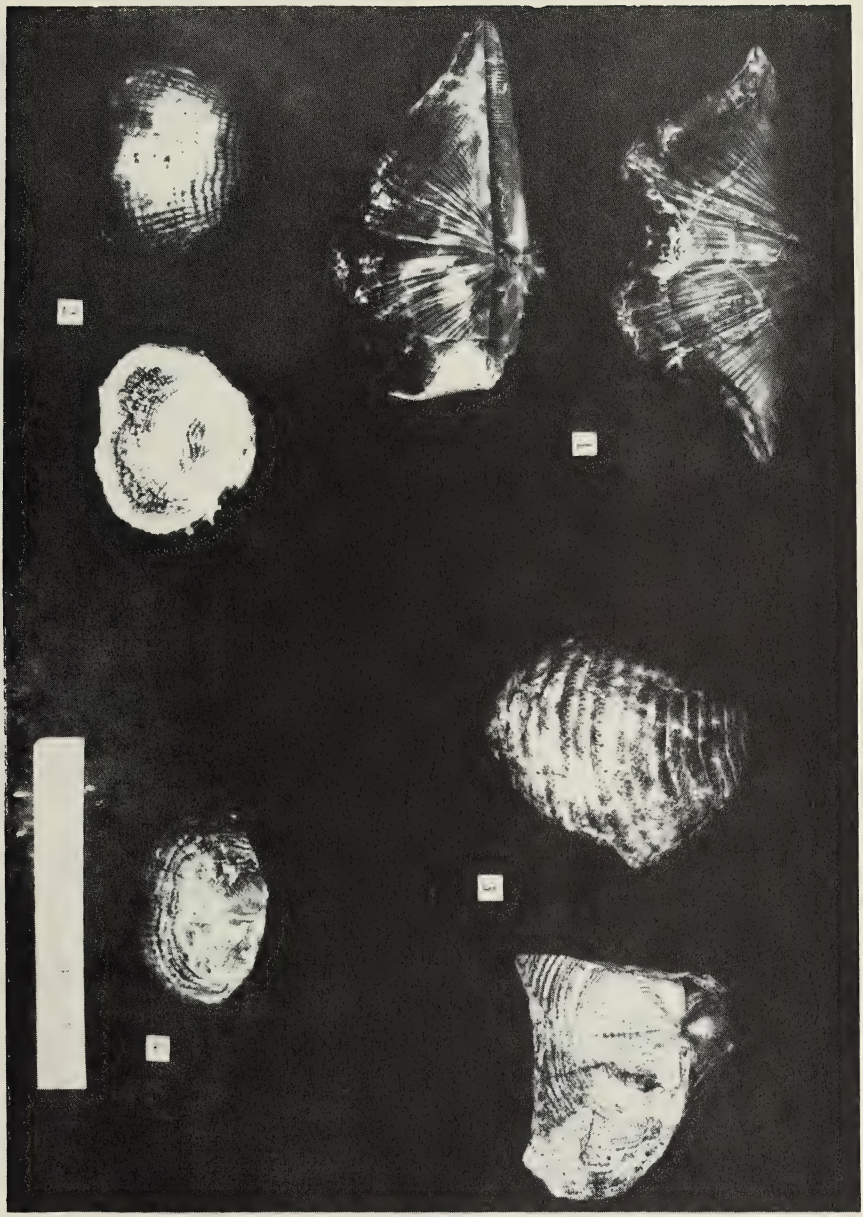

PLATE 4

FAUNA	LOCALITIES 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1. *Spirifera cameratus*	X X X
2. *Dictyoclostus portlockianus*	X X
3. *Echinoconchus semipunctatus*	X
4. *Dictyoclostus americanus*	X X

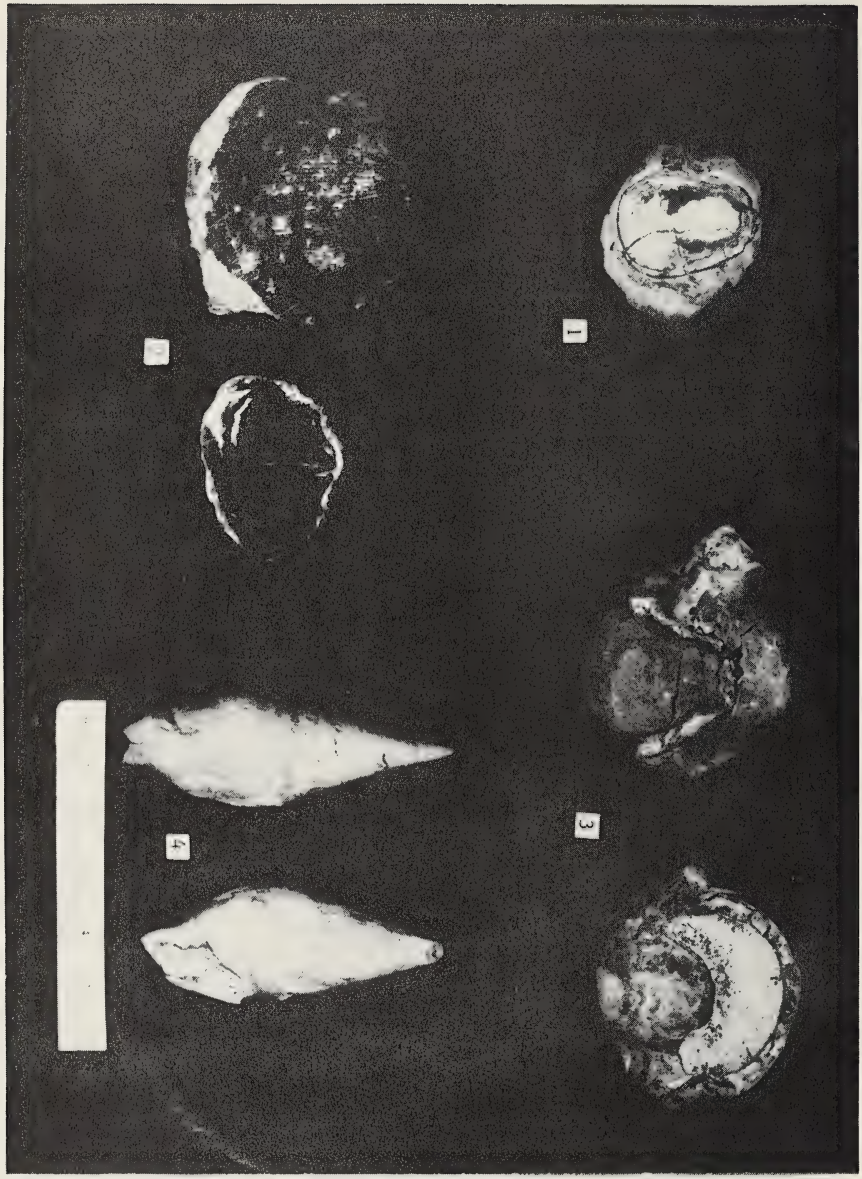

PLATE 5

FAUNA	LOCALITIES 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1. *Lingula sp.*	X
2. *Linoproductus prattenianus*	X X
3. *Euphemites* (?)	X X X
4. *Strobeus regularis*	X

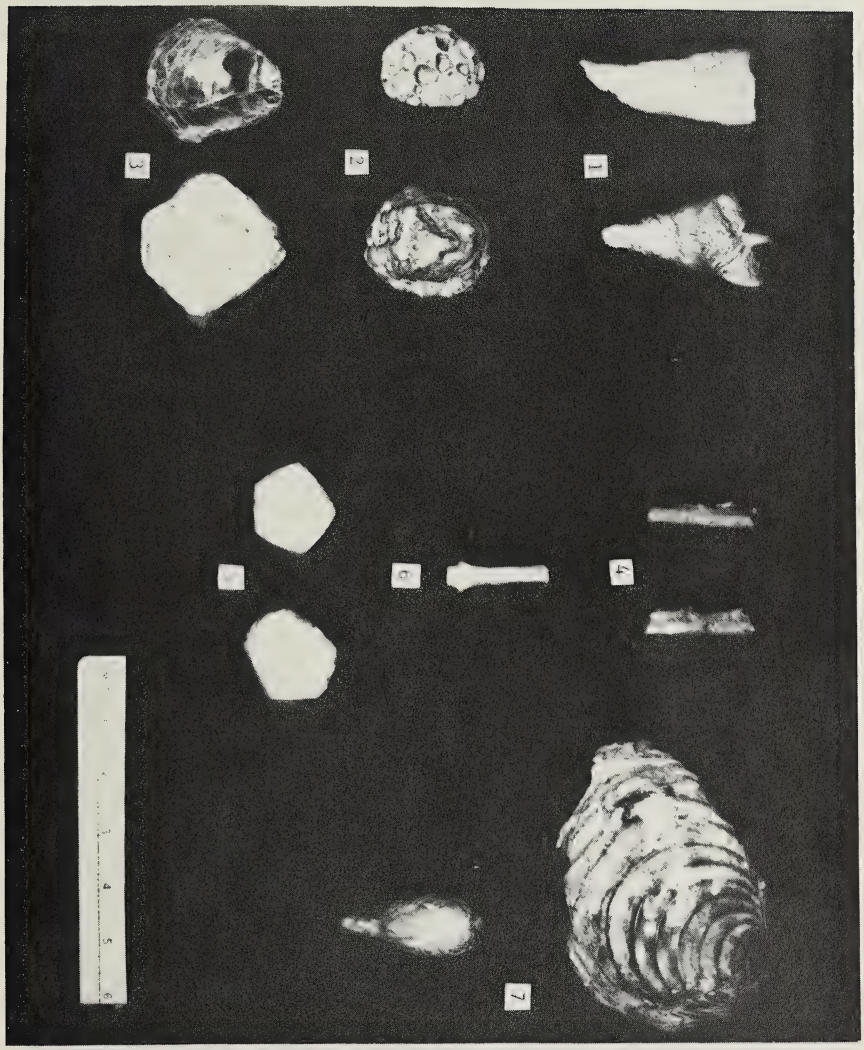

PLATE 6

FAUNA	LOCALITIES 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1. *Lophophyllidium profundum*	X X X
2. *Pleurodictyum*	X
3. *Composita subtilita*	X X
4. *Archeocidaris sp**	X
5. *Archeocidaris sp**	X
6. *Ulocrinus occidentalis***	X
7. *Allorisma sp.*	X X

*Echinoid spine
**Crinoid plate

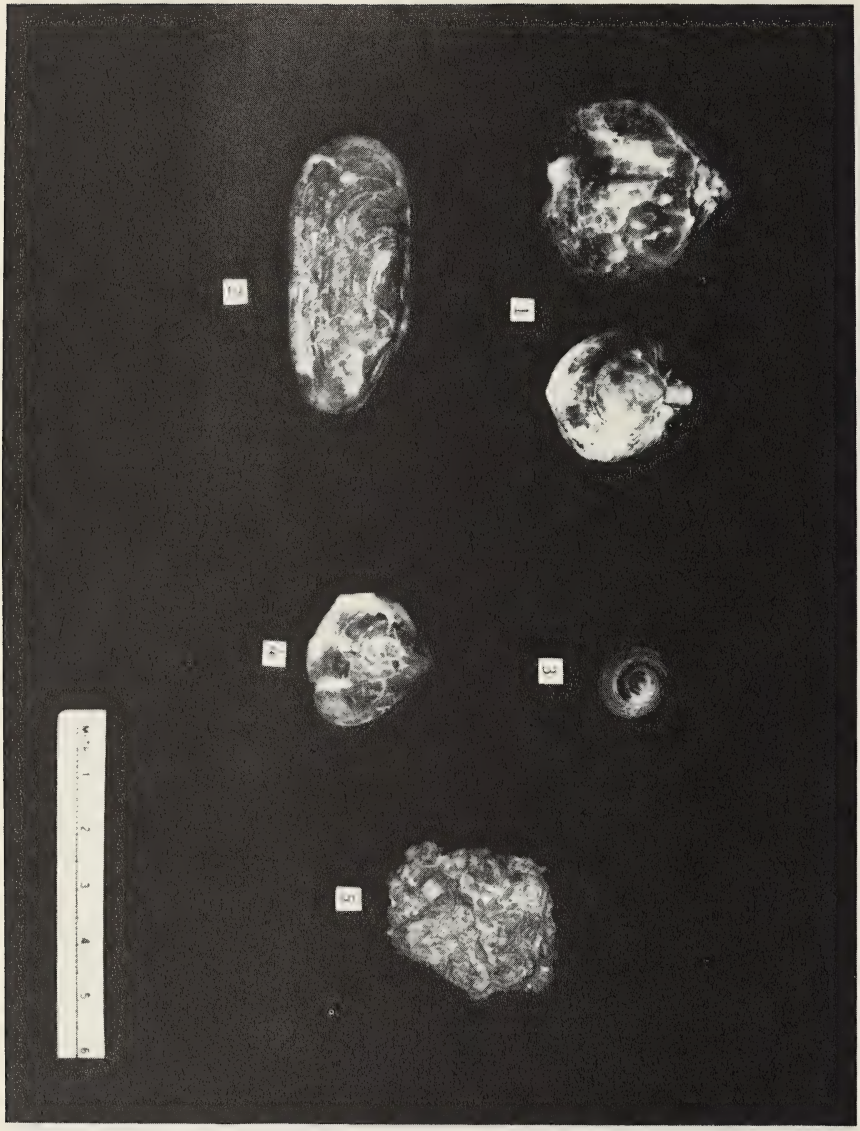

PLATE 7

FAUNA	LOCALITIES 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1. *Composita subtilita*	X X X
2. Unidentified Pelecypod	X
3. Unidentified Gastropod	X
4. Unidentified Pelecypod	X
5. Protozoa	X X

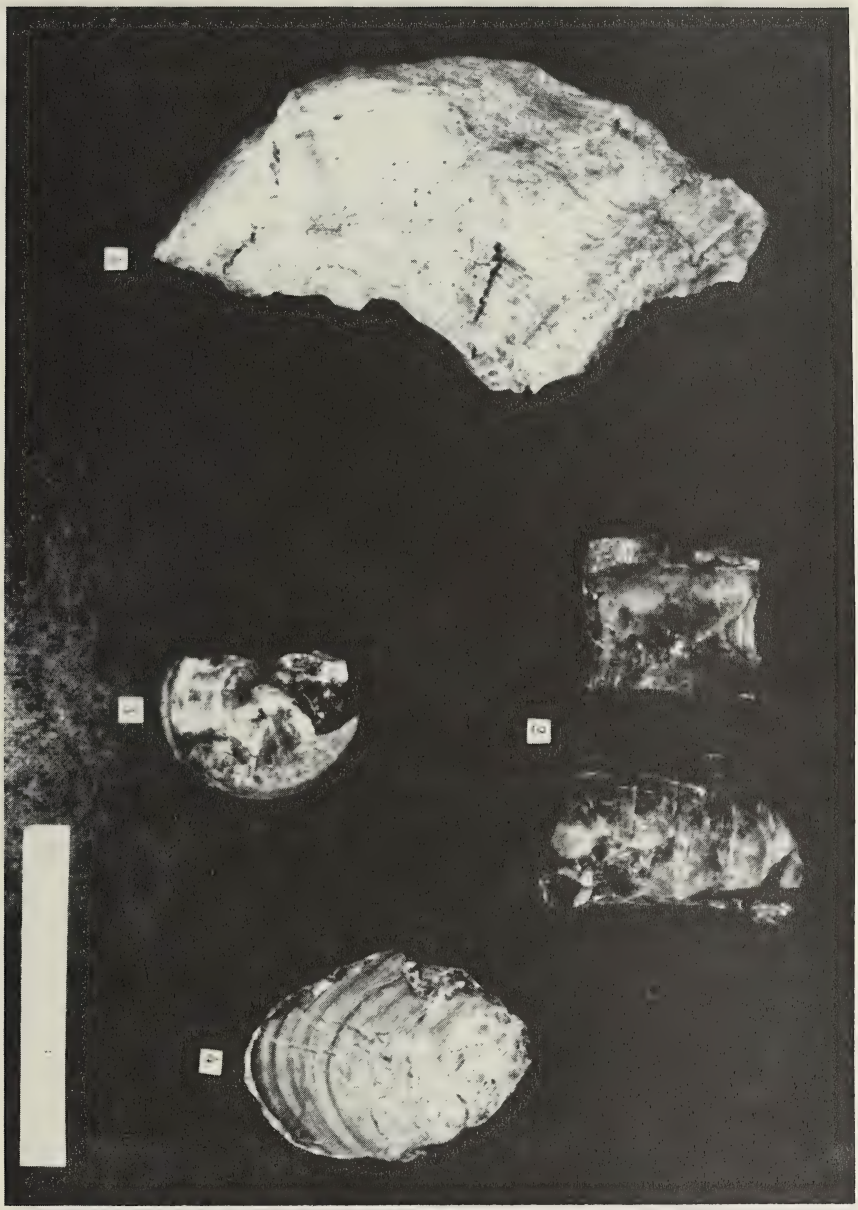

PLATE 8

FAUNA	LOCALITIES 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1. Unidentified coral	X
2. Unidentified straight Cephalopod	X
3. Unidentified fragment of Gastropod	X
4. Unidentified fragment of Pelecypod	X

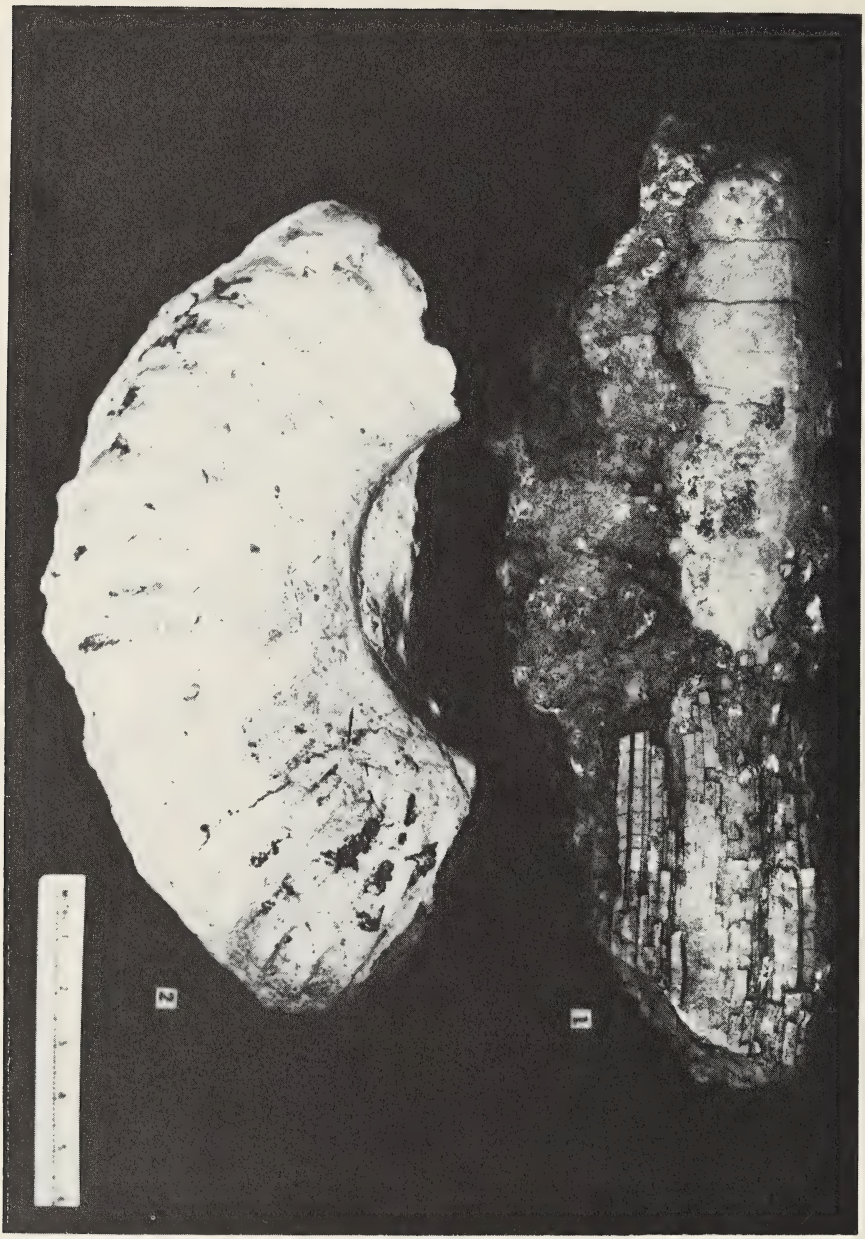

PLATE 9

FAUNA	LOCALITIES																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Caprina occidentalis*		X																		
2. *Oxytropidoceras belknapi*	X		X	X		X										X		X	X	X

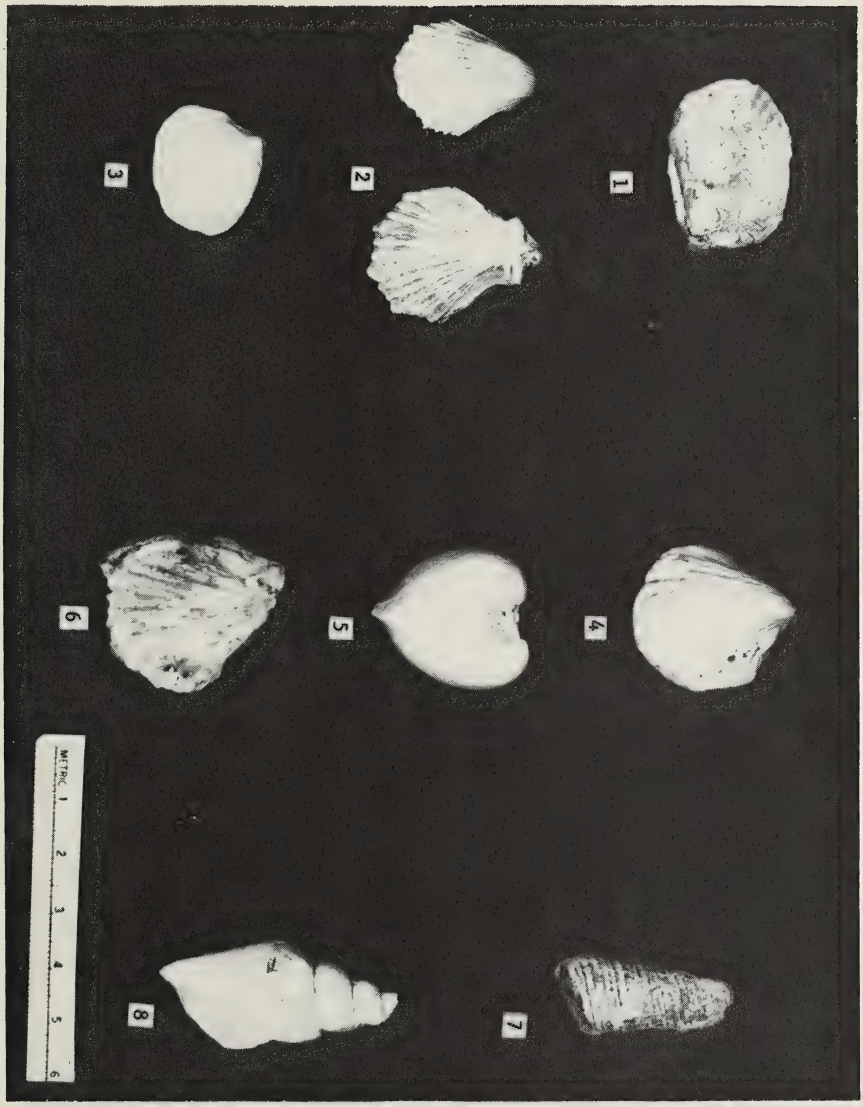

PLATE 10

FAUNA	LOCALITIES																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Lima wacoensis*	X		X	X	X	X		X		X						X			X	X
2. *Pecten texanus*	X		X	X		X										X			X	
3. *Meretrix sp.*	X		X	X		X										X				
4. *Cardium sp.*	X		X	X		X	X					X						X	X	
5. *Protocardia securiformis*	X		X	X	X	X		X				X				X			X	
6. *Pecten subalpina*	X		X	X												X			X	
7. *Turritella sp.*	X		X	X	X	X	X	X	X	X	X	X				X	X	X	X	X
8. *Turritella sp.*	X		X	X	X	X	X	X	X	X	X	X				X	X	X	X	X

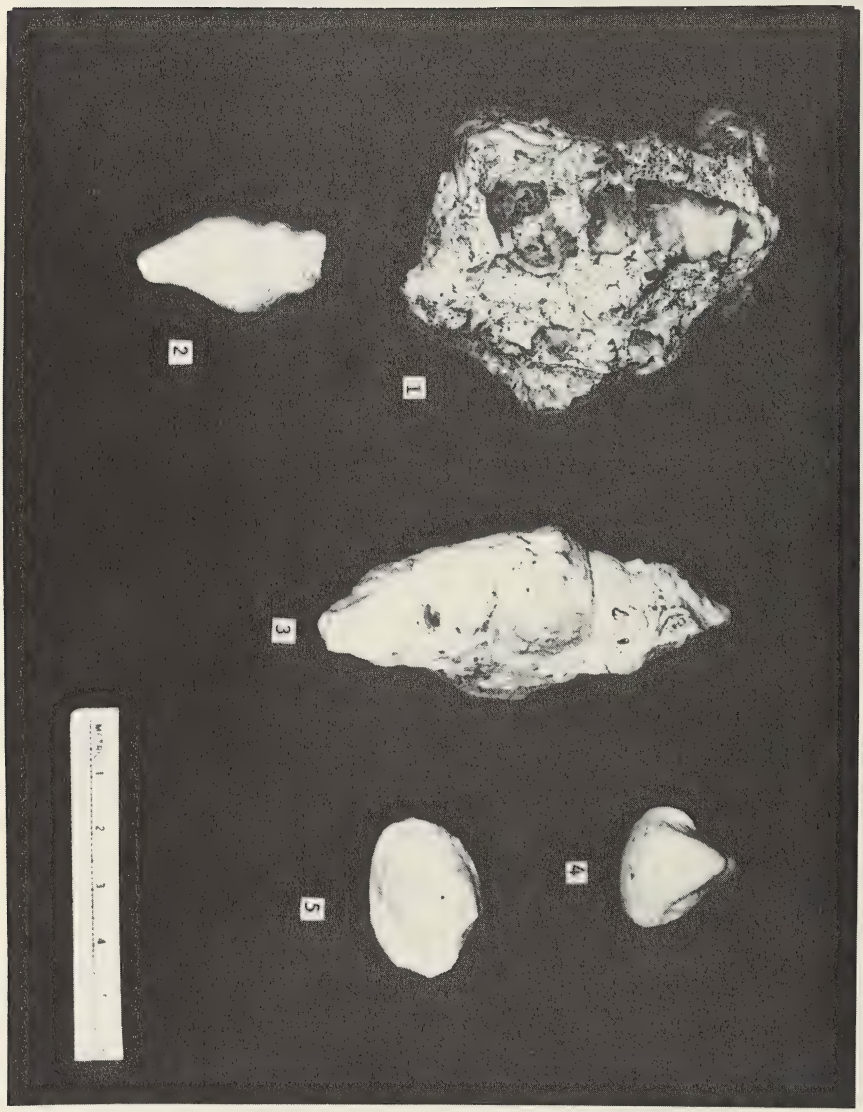

PLATE 11

FAUNA	LOCALITIES 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. Unidentified Gastropod																	X			
2. Unidentified Gastropod				X															X	
3. Unidentified Gastropod			X																X	
4. Unidentified Pelecypod	X			X																
5. Unidentified Pelecypod	X			X																

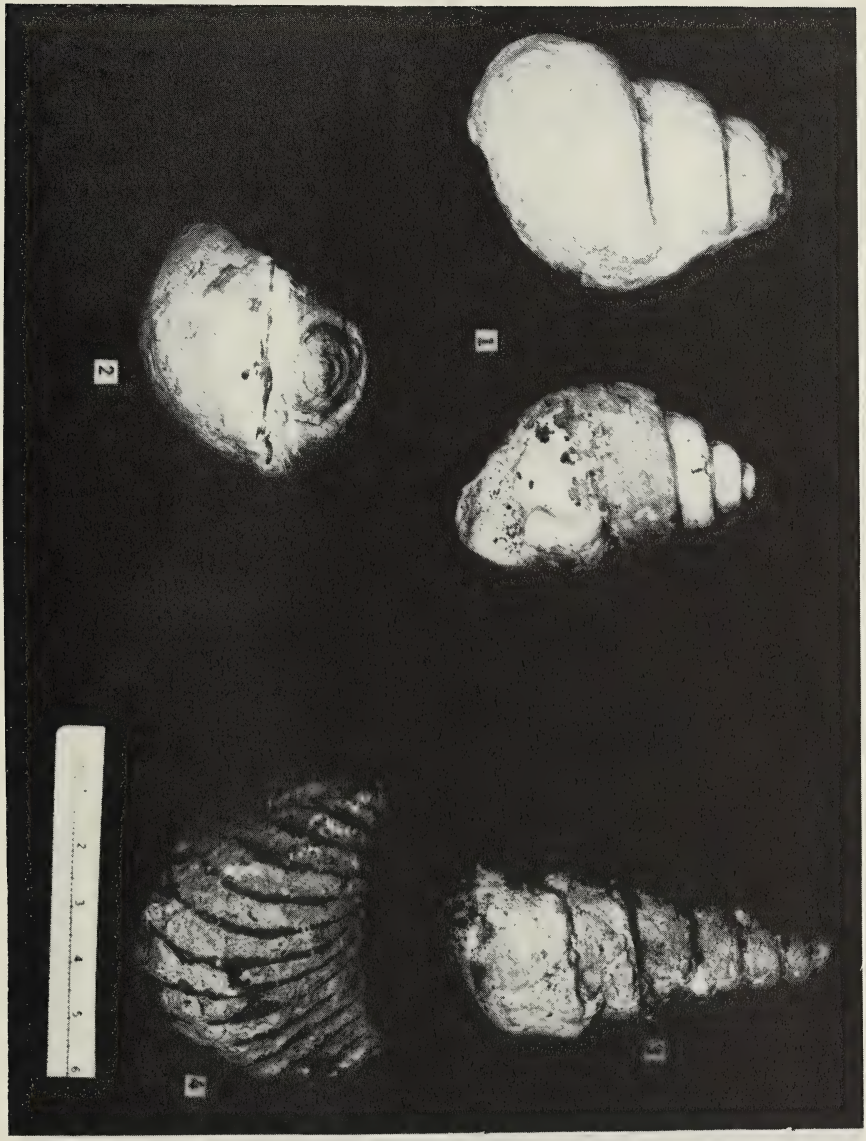

PLATE 12

FAUNA	LOCALITIES																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Tylostoma elevatum*	X		X	X					X									X	X	X
2. *Gyrodes sp.*	X																			
3. *Cerithium bosquenses*	X		X	X																
4. *Trigonia emoryi*	X		X													X				

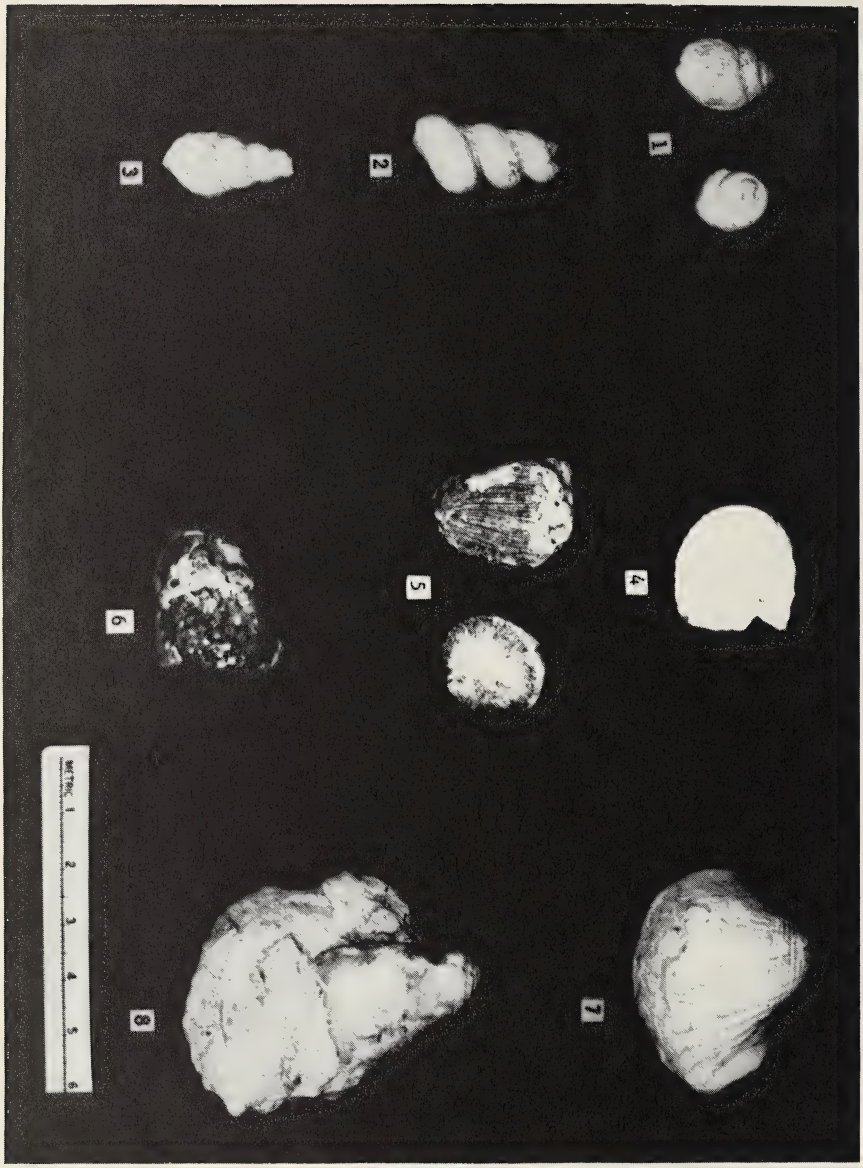

PLATE 13

FAUNA	LOCALITIES																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Lunatia halli*	X		X	X		X										X			X	
2. *Turritella sp.*	X		X	X		X		X	X			X				X			X	
3. Unidentified Gastropod			X																	
4. *Hamites fremonti*				X																
5. *Dungulia texana*	X		X	X				X			X					X		X	X	X
6. *Diploastrea harrisi*				X																
7. *Pholadomya sancti-sabae*	X		X	X	X			X								X			X	
8. *Crenella sp.*							X													

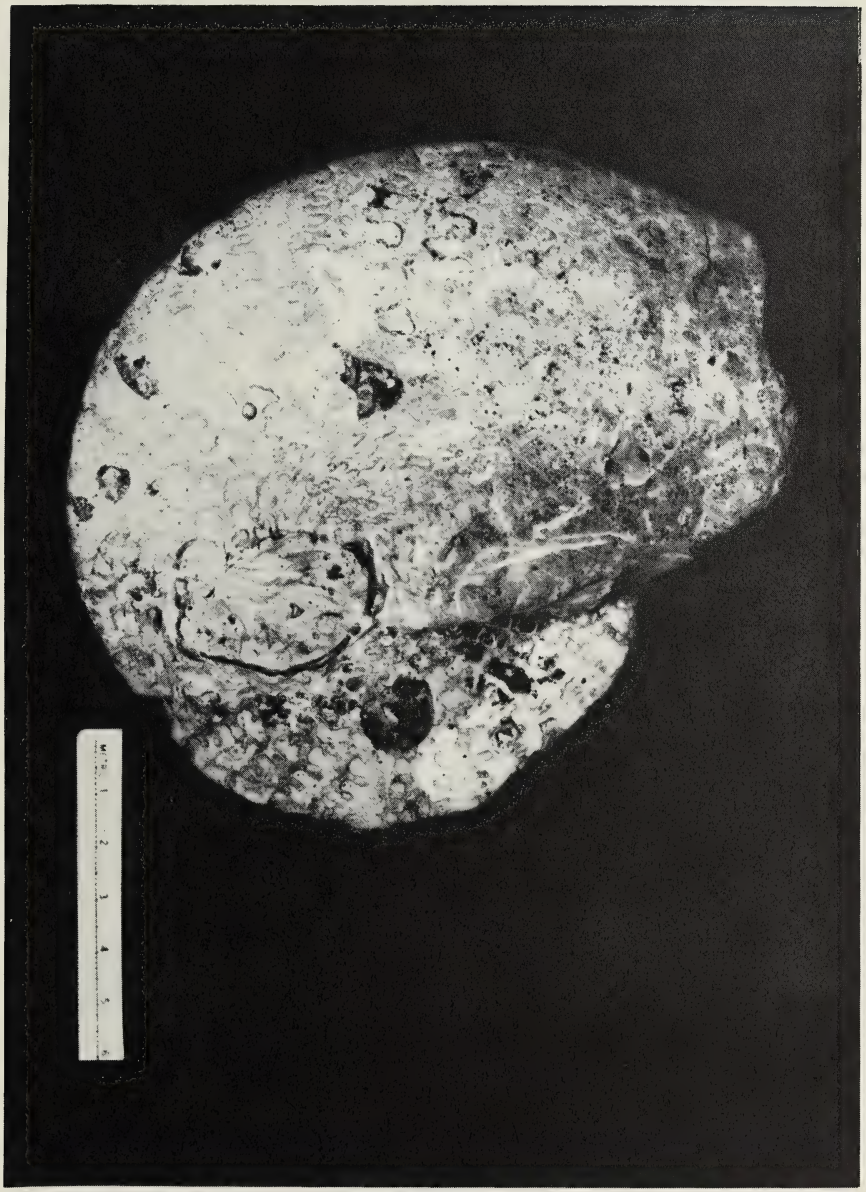

PLATE 14

FAUNA	LOCALITIES																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Metengonoceras*	X		X	X		X				X						X			X	

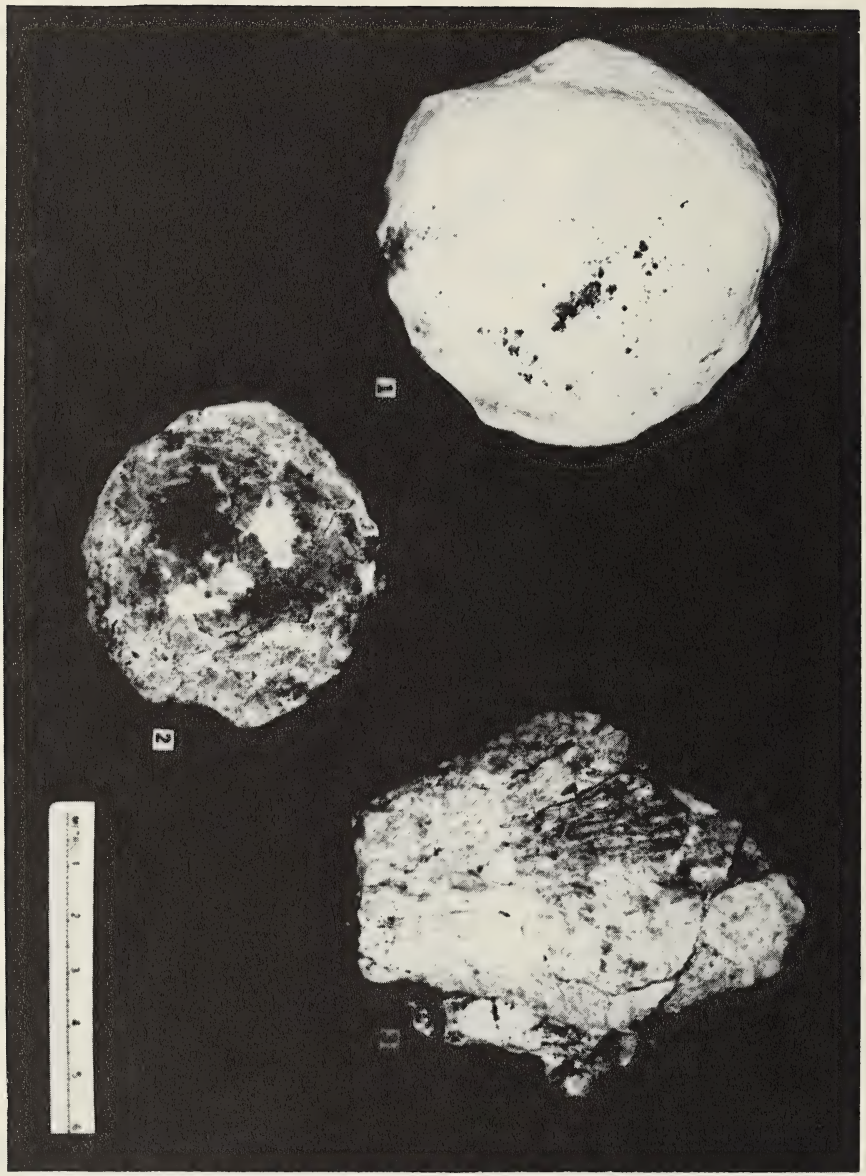

PLATE 15

FAUNA	LOCALITIES																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Cyprimeria gigantea*	X		X																	
2. *Cyprimeria texanus*	X		X			X										X				
3. *Ostrea sp.*		X																		

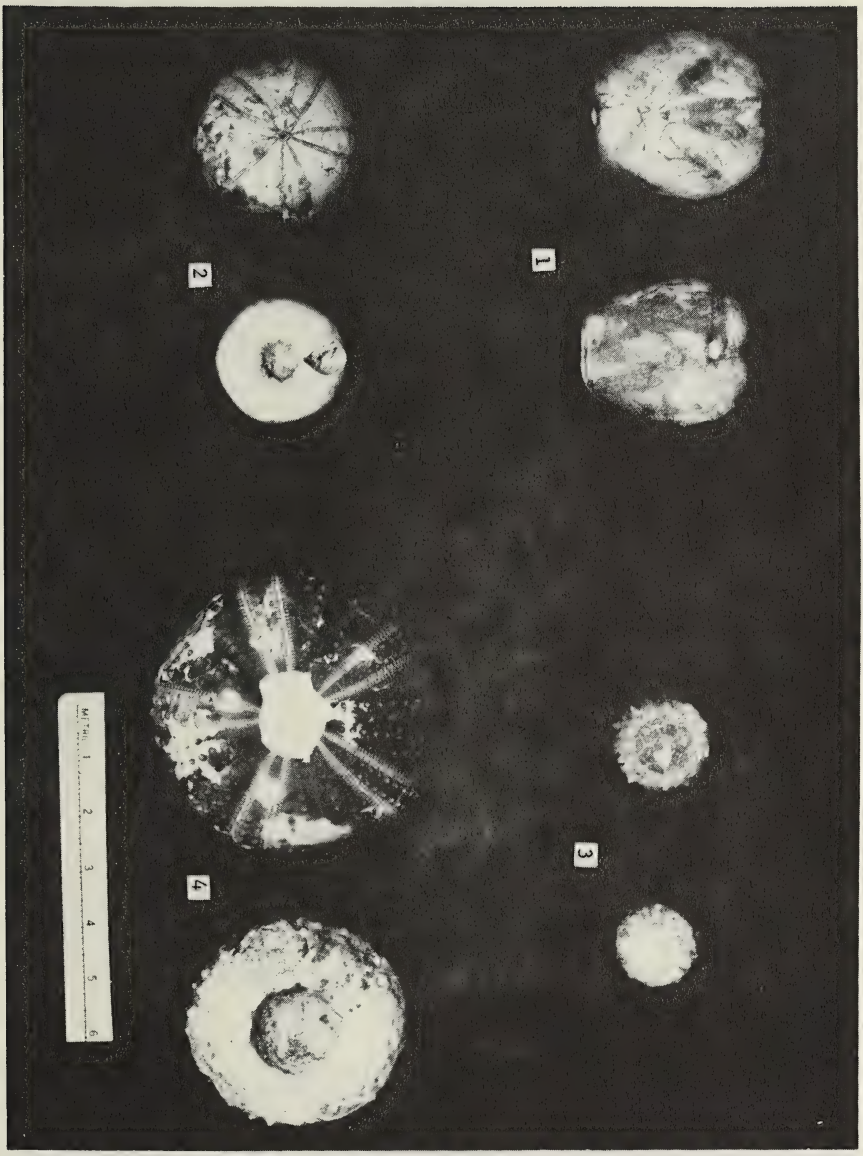

PLATE 16

FAUNA	LOCALITIES																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Ennalaster texana*	X		X	X		X			X		X					X		X	X	X
2. *Holectypus planatus*	X		X			X														
3. *Salenia texana*	X		X	X																
4. *Phymosoma texanum*	X		X	X												X			X	

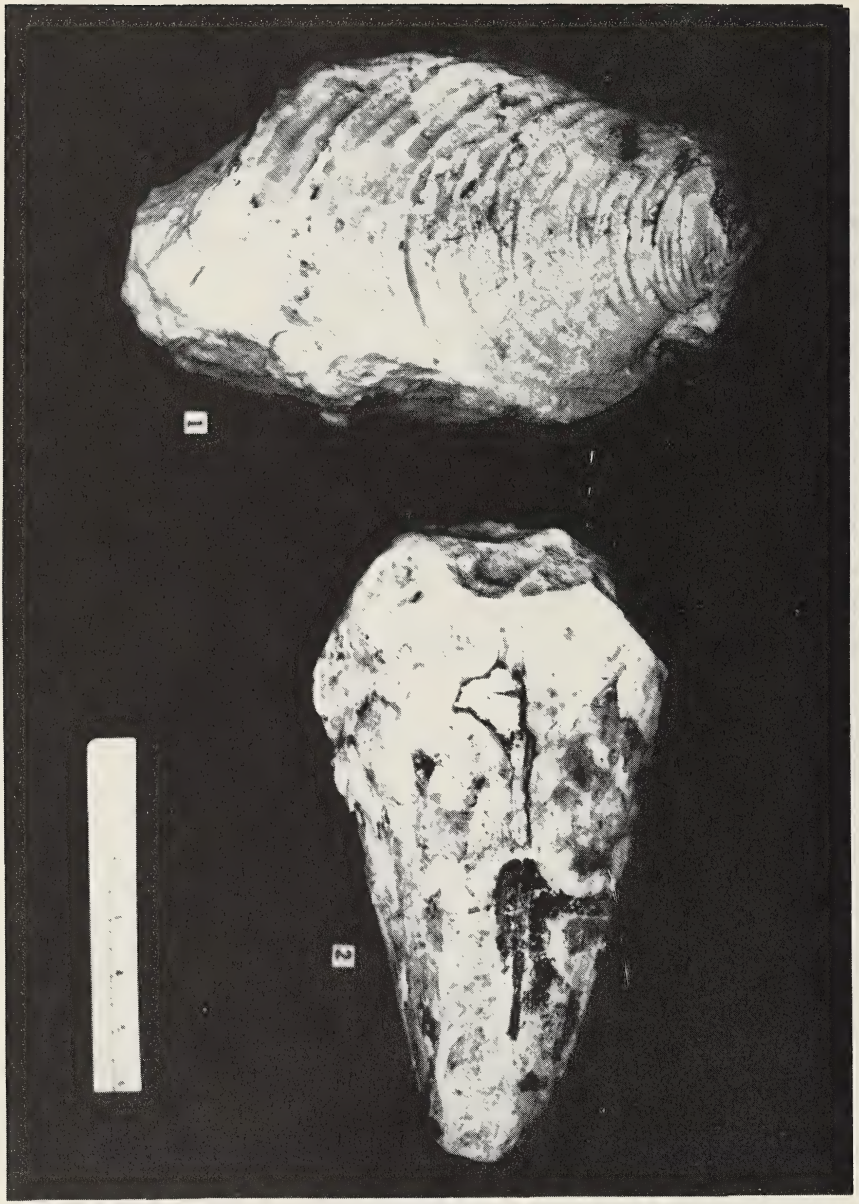

PLATE 17

FAUNA	LOCALITIES 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Inoceramus comanchensis*	X		X																	
2. *Pinna comancheana*	X		X																	

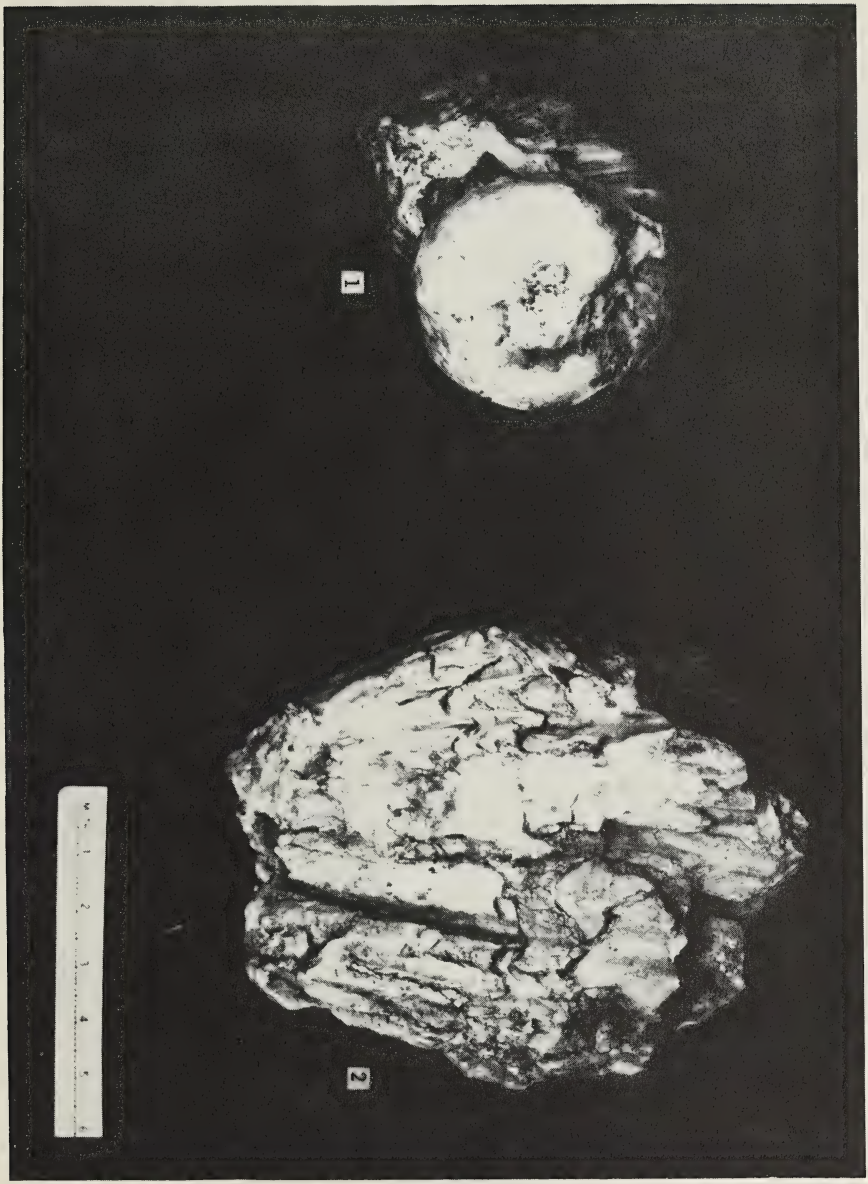

PLATE 18

FAUNA	LOCALITIES 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1. *Toucasia texana*	X
2. *Radiolites davidsoni*	X

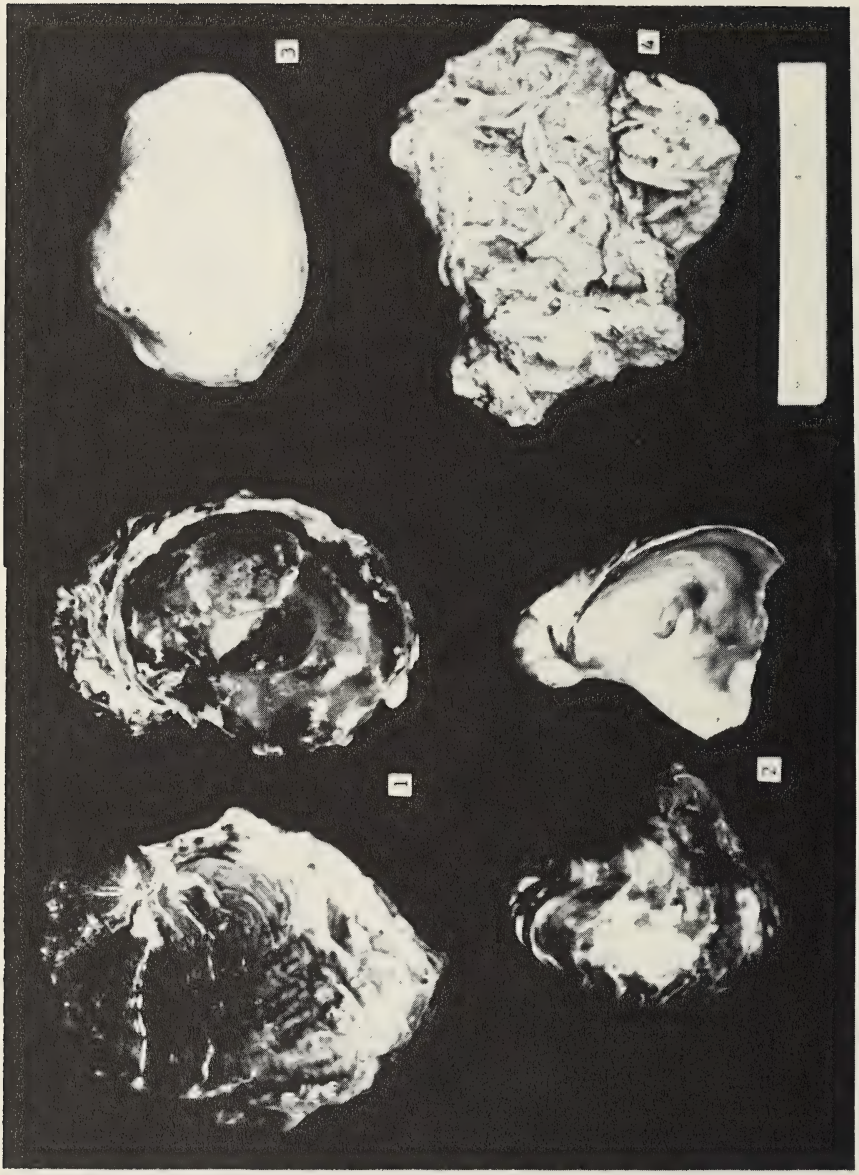

PLATE 19

FAUNA	LOCALITIES																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Exogyra texana*	X		X	X	X	X		X	X	X	X	X				X		X	X	X
2. *Gryphaea navia*	X		X	X	X	X		X	X	X	X	X				X		X	X	X
3. *Homomya tarrantensis*	X		X	X							X					X		X	X	
4. *Serpula pervermiformis*	X		X	X	X	X	X	X		X						X		X	X	

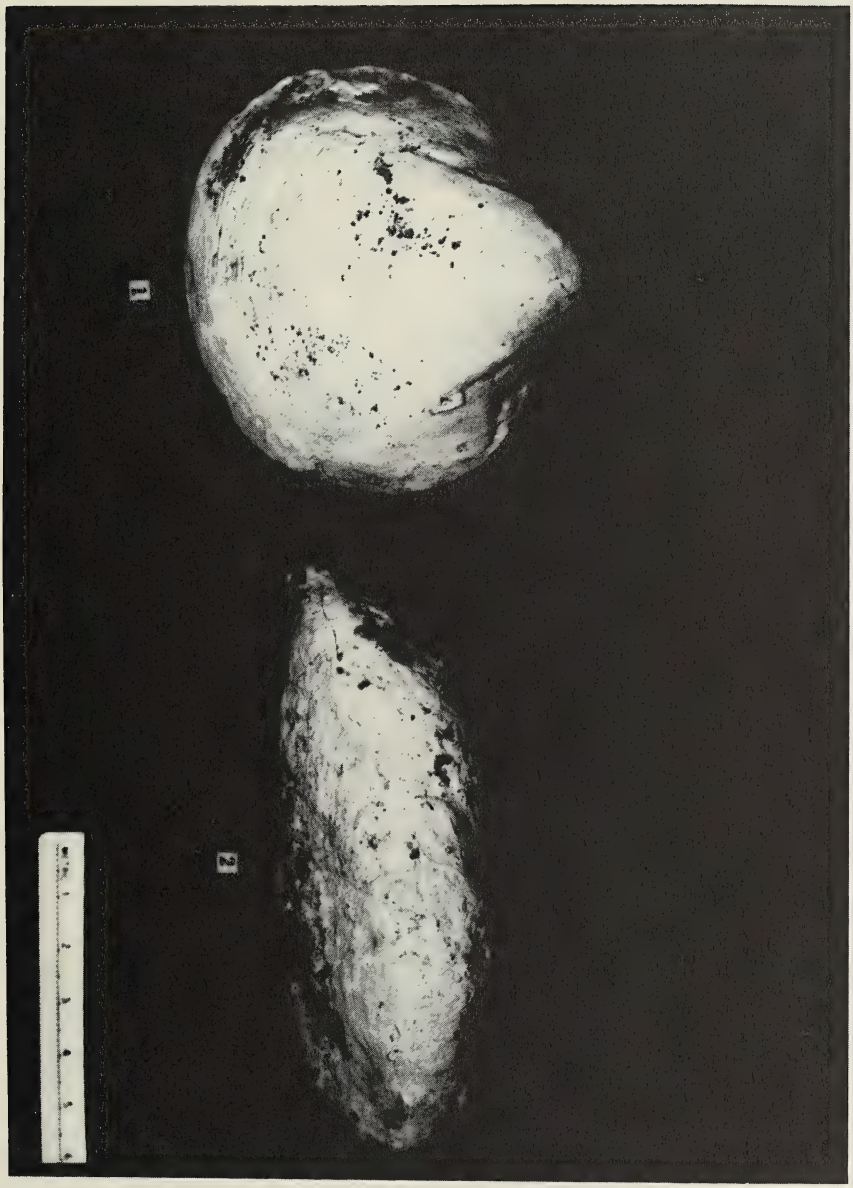

PLATE 20

FAUNA	LOCALITIES																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Protocardia texana*	X		X	X	X	X	X	X	X	X						X		X	X	X
2. *Leptosolen sp.*	X																			

PLATE 21

PLATE 22

ACKNOWLEDGEMENTS

The author wishes to acknowledge the cooperation of the landowners who permitted access to collecting sites on their property. In particular Mr. William Anderson, Route 1, Hico, Texas; Mr. Randy Bennett of the Thurber Ranch; Mr. Wayne York, Patillo, Texas; and Mr. T. T. Lowe of Santo, Texas.

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MORPHOMETRIC AND MERISTIC CHARACTERS OF THE WHITE BASS, *MORONE CHRYSOPS*, FROM THE RED AND WASHITA RIVER SEGMENTS OF LAKE TEXOMA[1]

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Miami, Florida 33149

ABSTRACT

Morphometric characters were measured on 335 white bass, *Morone chrysops*, 171 from the Red River and 164 from the Washita River segments of Lake Texoma. Regressions of 7 characters on total length were analyzed by covariance analysis. Five of these could be used to separate Lake Texoma white bass spawning aggregations. Also, the meristic characters studied consisted of counts made from the anal and left pectoral fin rays of 60 white bass from the Red River and 73 from the Washita River. The only difference was shown by the number of pectoral rays of 1 year class.

INTRODUCTION

The white bass, *Morone chrysops,* is one of the most abundant sport fishes present and probably the fish most frequently taken by anglers in Lake Texoma (Riggs and Bonn, 1959). As exploitation of this species increases, it is important to know whether the sport fishery is exploiting a general lake population or discrete subgroups so that fishery management procedures can be implemented.

White bass were collected just prior to the spawning season of 1970 and 1971 from Lake Texoma, an impoundment of the Red and Washita Rivers. This artificial lake was filled in 1944 and it is located along the Oklahoma-Texas border. Since white bass spawn in both the Red and Washita Rivers, this occurrence suggested that 2 separate spawning aggregations might exist. Because it has been suggested that the white bass has a well-developed homing behavior (Horrall, 1961), a spawning isolating mechanism is herein postulated, which could lead to these separate spawning groups.

Morphometic and meristic comparisons have been used for separating subpopulations. These comparisons were used on striped bass, *Morone saxatilis,* from various river systems (Lund, 1957; Lewis, 1957). This paper examines the usefulness of these types of comparisons for showing heterogeneity within the white bass from Lake Texoma.

[1] This study was part of a dissertation presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Oklahoma.

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MATERIALS AND METHODS

Morphometric Characters

A total of 335 fish was collected from the rivers just before the spawning seasons in 1970 and 1971. The ratio of males to females was about equal for the samples from each river. The characters used were: least depth of caudal peduncle, distance between insertion of left pectoral fin and origin of spinous dorsal fin, left pectoral fin length, body depth, predorsal length, head length, and vertical distance between lateral line and origin of spinous dorsal fin. All measurements were made to the nearest mm. Regression equations were calculated for the above measurements, against total length and an analysis of covariance was used to analyze the results.

Meristic Characters

Anal and left pectoral ray counts were made on 133 fish; 60 from the Red River and 73 from the Washita River, taken just before the spawning seasons in 1970 and 1971. An analysis of variance was conducted on these data.

RESULTS

Morphometric Characters

Each of the morphometric comparisons when plotted, showed a linear relation. The regression lines obtained from the least squares method are plotted in Figs. 1 and 2.

Analysis of covariance was used to determine if there was any significant difference in slopes at the 0.01 probability level. Significant differences in regression coefficients were found between the spawning aggregations from the 2 rivers for least depth of caudal peduncle, pectoral-dorsal distance, predorsal length, body depth, and lateral line to dorsal distance, all plotted against total length. There were no significant differences for regression coefficients between the 2 localities for both head and left pectoral fin lengths plotted against total length. Because there were no significant differences found for these 2 characters, it was possible to test for the adjusted mean F. The 0.01 level of significance was also used for this test. Total length to head and left pectoral lengths were not significantly different between localities. The regression equations, with standard error and other quantities necessary for determination of confidence limits, are shown in Table 1

Meristic Characters

Rays in the anal and left pectoral fins were counted for each fish studied. Analysis of variance at the 0.01 probability level was used to determine if any significant differences were present (Table 2).

No significant difference was found in anal fin ray counts between fish from the 2 areas. The mean anal ray count was 15.85 for the Red River and 15.67 for the Washita River white bass.

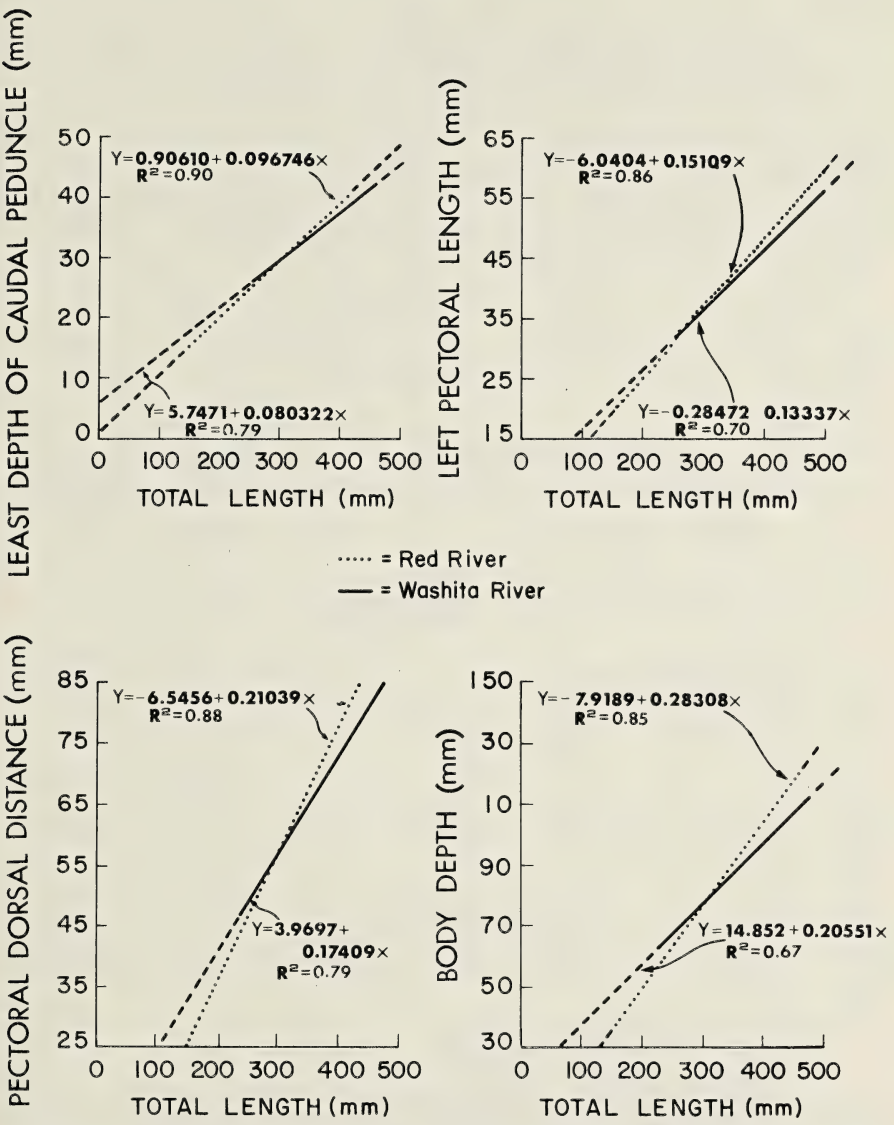

Figure 1. Regression lines of least depth of caudal peduncle, left pectoral length, pectoral-dorsal distance, and body depth on total length of white bass by locality.

A significant difference in the number of rays in the left pectoral fin at the 0.01 level was found between fish from the 2 areas (Table 2). There was no significant difference in the left pectoral fin ray counts between the 1967, 1968, and 1969 year classes of Red River white bass, and none between the 1967, 1968, and 1969 year classes from the Washita River (Table 3). Between the Red and Washita rivers 1968 year classes, a significant difference in the left pectoral fin ray counts was found, however, no significant difference was found between

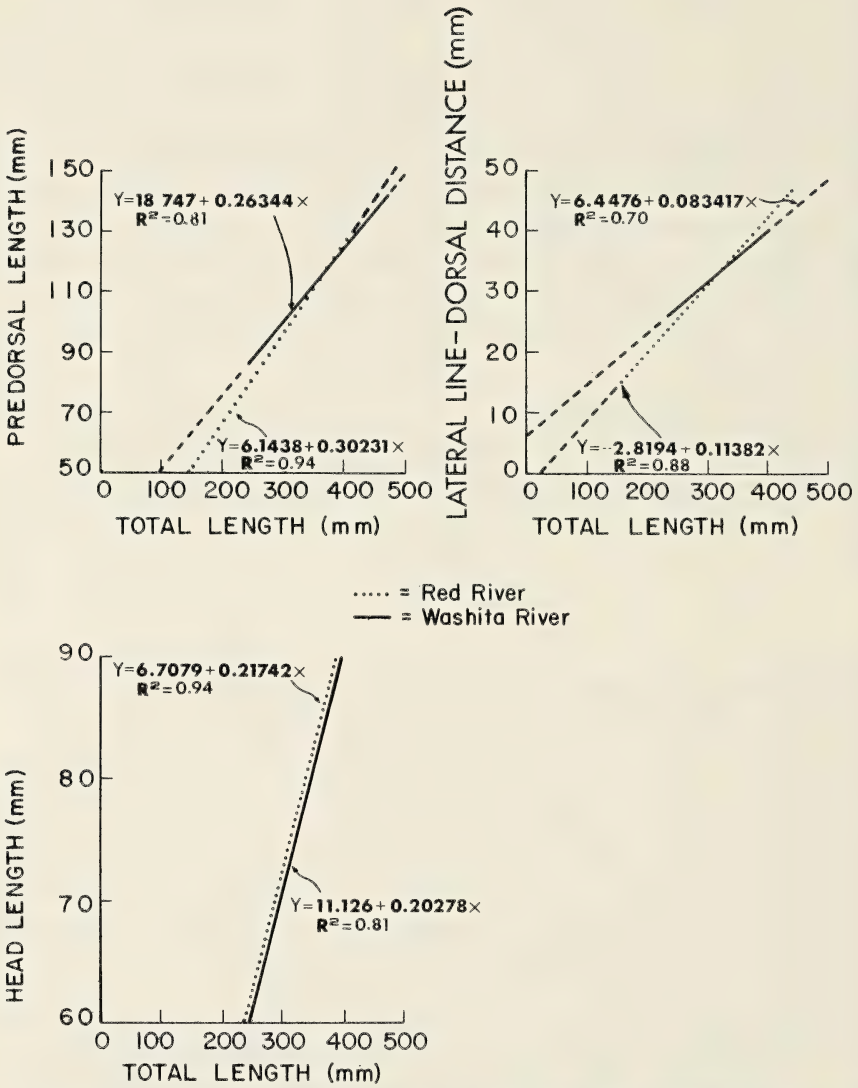

Figure 2. Regression lines of predorsal length, lateral line-dorsal distance, and head length on total length of white bass by locality.

the 1967 and 1969 year classes of each area. The mean left pectoral fin ray count for fish from the Red River 1967 year class was 16.00. In the 1968 year class it was 15.53, and in the 1969 year class it was 15.47. The mean count for the left pectoral fin for the Washita River fish was 16.18 for the 1967 year class, 16.00 for the 1968 year class, and 15.78 for the 1969 year class. The mean count for the left pectoral fin rays was consistently higher for white bass from the Washita River compared to similar counts from the Red River; it was only significantly higher for the 1968 year class.

TABLE 1

The Regression Equations, Unexplained Mean Square, Sum of Squares of x, Number, and Standard Error Calculated for 7 Morphometric Characters on Total Length of White Bass from the Red and Washita River Arms of Lake Texoma

Locality	$Y = \bar{Y} + b(X - \bar{X})$	$S^2_{Y \cdot X}$	Σx^2	N	S_b
Total length – least depth of caudal peduncle					
Red River	29.743 + 0.096746(X - 298.064)	2.350	371,104.292	171	0.002
Washita River	31.720 + 0.080322(X - 323.354)	1.731	167,803.487	164	0.003
Total length – pectoral-dorsal distance					
Red River	56.164 + 0.21039(X - 298.064)	11.900	371,104.292	171	0.006
Washita River	60.262 + 0.17409(X - 323.354)	8.247	167,803.487	164	0.007
Total length – head length					
Red River	71.515 + 0.21742(X - 298.064)	7.274	371,104.292	171	0.004
Washita River	76.695 + 0.20278(X - 323.354)	9.586	167,803.487	164	0.008
Total length – left pectoral length					
Red River	38.994 + 0.15109(X - 298.064)	8.150	371,104.292	171	0.005
Washita River	42.842 + 0.13337(X - 323.354)	7.389	167,803.487	164	0.007
Total length – predorsal length					
Red River	96.251 + 0.30231(X - 298.064)	14.477	371,104.292	171	0.006
Washita River	103.933 + 0.26344(X - 323.354)	17.322	167,803.487	164	0.010
Total length – body depth					
Red River	76.456 + 0.28308(X - 298.064)	30.159	371,104.292	171	0.009
Washita River	81.304 + 0.20551(X - 323.354)	20.800	167,803.487	164	0.011
Total length – lateral line to dorsal distance					
Red River	31.105 + 0.11382(X-298.064)	3.626	371,104.292	171	0.003
Washita River	33.421 + 0.083417(X - 323.354)	2.940	167,803.487	164	0.004

TABLE 2

Analysis of Variance of Meristic Characters of White Bass Collected from Both the Red and Washita River Segments of Lake Texoma

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	F
Anal Fin				
Between groups	1.052	1	1.052	2.0
Within groups	67.760	131	0.517	
TOTAL	68.812	132		
Left Pectoral Fin				
Between groups	4.793	1	4.793	14.4*
Within groups	43.508	131	0.332	
TOTAL	48.301	132		

*Significant at the 0.01 level.

TABLE 3

Analysis of Variance of the Mean Number of Soft Rays in the Left Pectoral Fin for 1967, 1968, and 1969 Year Classes of White Bass from the Red and Washita Rivers

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	F
Left Pectoral for Red River, 1967, 1968, 1969				
Between groups	1.137	2	0.568	1.5
Within groups	21.208	55	0.386	
TOTAL	22.345	57		
Left Pectoral for Washita River, 1967, 1968, 1969				
Between groups	1.548	2	0.774	2.8
Within groups	19.105	69	0.277	
TOTAL	20.653	71		
Left Pectoral for Red River vs Washita River 1967 year class				
Between groups	0.114	1	0.114	0.4
Within groups	3.636	14	0.260	
TOTAL	3.750	15		
Left Pectoral for Red River vs Washita River 1968 year class				
Between groups	3.466	1	3.466	10.3*
Within groups	20.471	61	0.336	
TOTAL	23.937	62		
Left Pectoral for Red River vs Washita River 1969 year class				
Between groups	1.128	1	1.128	3.4
Within groups	16.206	49	0.331	
TOTAL	17.334	50		

*Significant at the 0.01 level.

DISCUSSION

What actually caused the differences in the body proportions of the white bass is subject to speculation. Hubbs (1941) said that some changes in body form are likely to have a genetic cause. Martin (1949) has shown that early development is important in determination of the relative size of body parts. He has also shown that temperature and diet during the early growth period yielded differences in body form. Therefore, the environmental conditions present when the fish is in its early stage of development, may have an effect on morphometric

characters. Wilder (1952) reported that populations of brook trout, *Salvelinus fontinalis,* reared at higher temperatures had larger body parts. Even though the reason for the differences is unknown, this investigation has demonstrated that they can be used as indicators for identifying separate spawning aggregations of white bass present in LakeTexoma.

Of the meristic characters studied, only the left pectoral ray counts were significantly different between the 2 spawning aggregations. Krumholz and Cavanah (1968) found stability among all the characters that they studied for the freshwater drum, *Aplodinotus grunniens,* from the lower Ohio River, Kentucky, and Lake Winnebago, Wisconsin. This stability has existed since early postglacial times. When studying vertebrae numbers in the yoke darter, *Etheostoma juliae,* Hill (1968) found significant differences between fish taken from the same river. No published reports on the meristic characteristics of white bass could be found; therefore, it is not known what amount of meristic stability is present in this species.

When comparing the left pectoral counts for different year classes from both rivers, a significant difference was revealed between only the 1968 year class from each river. This difference seems to indicate that changes in water temperature, or perhaps other factors, such as amount of salinity or other chemical conditions, occurred during larval life at the time the pectoral fin ray number was determined.

Low temperatures, high salinity, and low oxygen tensions result in longer development periods, which usually produce high fin ray counts (Tåning, 1952). Hubbs (1955) reported that the dorsal and anal fin ray numbers in subpopulations of mosquito fish, *Gambusia affinis,* are determined by the action of a single gene. Gabriel (1944) has shown a genetic basis for meristic variation in killifish, *Fundulus heteroclitus.*

This study has shown that some heterogeneity is present among the white bass of the Red and Washita River segments of Lake Texoma. However, the causes of these differences are unknown.

ACKNOWLEDGEMENTS

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A REVIEW OF PHYTOPLANKTON STUDIES IN LAKE LIVINGSTON, TEXAS

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ABSTRACT

Several phytoplankton-related studies in Lake Livingston, Texas have been conducted over the last several years, primarily in an effort to aid in the study of eutrophication phenomena in the reservoir. These studies have been of 2 general types: primary productivity measurements and limiting nutrient studies. Primary productivity studies have utilized chlorophyll *a*, cell enumerations, and ^{14}C as means of measuring phytoplankton abundance and/or productivity. Limiting nutrient tests have all been variations of the nutrient spike with emphasis on the elements nitrogen and phosphorus. All previous phytoplankton studies in Lake Livingston are herein reviewed and summarized.

INTRODUCTION

Lake Livingston, a joint project of the City of Houston and the Trinity River Authority, is located on the Trinity River in Polk, San Jacinto, Trinity, and Walker Counties, with headwater portions in Houston, Leon, and Madison Counties (see Fig. 1). Lake Livingston dam was completed in August 1969. The reservoir has a normal pool level of 131 ft (40 m) MSL and a storage capacity of 1.75×10^6 acre-ft (2.16×10^9 m^3). It has a surface area of 82,600 acres (33,400 ha) and a drainage area of approximately 16,716 mi^2 (4.33×10^6 ha). The mean depth of the reservoir is 20.6 ft (6.3 m). The main body of Lake Livingston is about 30 mi (48 km) in length.

Since impoundment was completed in November 1971, Lake Livingston has exhibited numerous eutrophic symptoms, due primarily to the high nutrient input from the Trinity River. These eutrophic symptoms include anoxic summertime hypolimnia, large diurnal swings in dissolved oxygen, and proliferation of phytoplankton and macrophytes. Phytoplankton studies conducted in Lake Livingston have been directed at (1) determining algal intensity and/or productivity and (2) determining the limiting factor(s) affecting algal growth and development in the reservoir. A number of tests presently exist for determining these phytoplankton-nutrient relationships (Reid, 1961; Maloney, *et al.*, 1972; Powers, *et al.*, 1972; EPA, 1973; APHA, 1975; and Lane and Levins, 1977), and many have been applied to the phytoplankton community in Lake Livingston.

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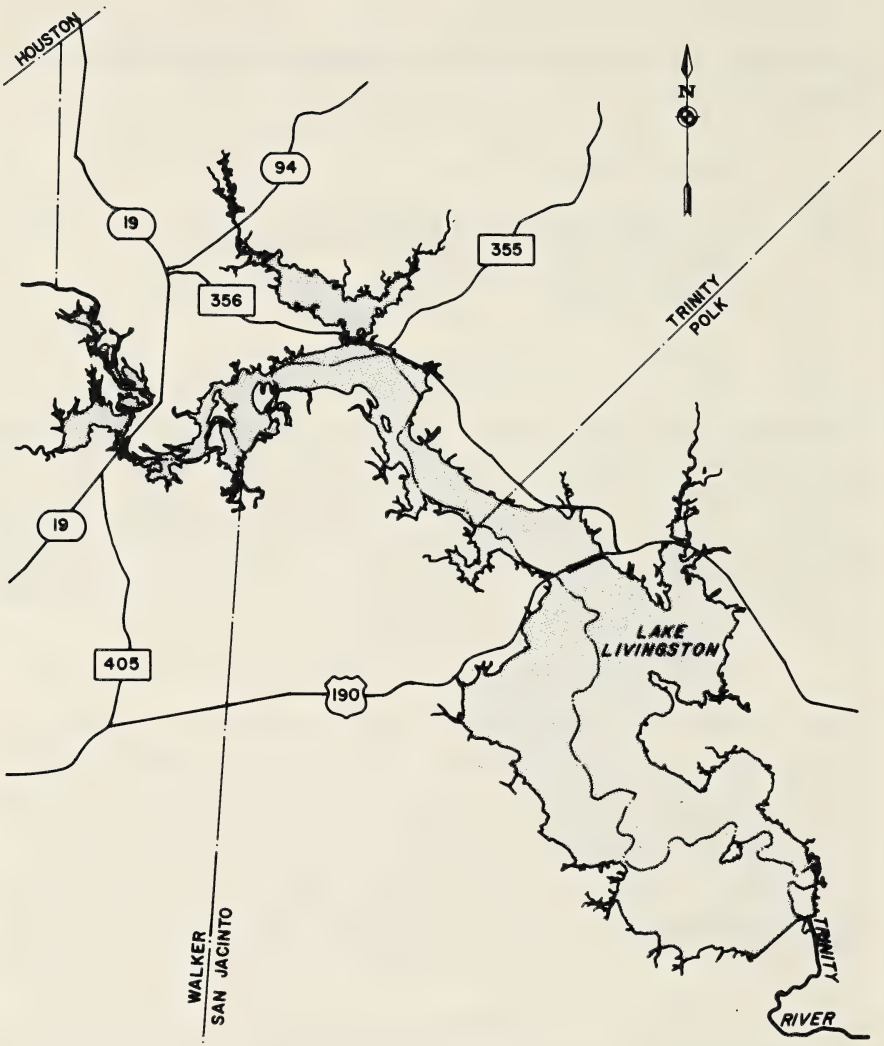

Figure 1. Map of Lake Livingston.

The primary purpose of this survey is to present a synoptic review of the various phytoplankton-related studies which have been conducted in Lake Livingston. It is the hope of the author that this compilation will aid continuing study into the degree of and possible control of eutrophication phenomena in Lake Livingstson.

PRODUCTIVITY STUDIES

A variety of techniques are available for determining the phytoplankton productivity of a body of water, including changes with respect to time in chlorophyll concentration, cell enumeration, cell volume, and standing crop (biomass).

Also included in the list of productivity methods are production of oxygen over a given time period, uptake of carbon dioxide (including the use of ^{14}C-labeled carbonate) over a given time period, and uptake of nutrients (including the use of ^{32}P, etc.) over a given time period. The significance of the primary production measurement lies in the fact that it represents a majority of the organic synthesis occurring within a particular aquatic ecosystem.

Tests for both the production of oxygen and the uptake of carbon employ the "light and dark bottle" technique, whereby water samples are suspended *in situ* in the water column. One sample container is clear, measuring both photosynthesis and respiration, and one is opaque, measuring only respiration. The dissolved oxygen method may provide estimates of both net and gross photosynthetic activity, while the ^{14}C method provides an estimate most nearly approximating net photosynthesis. Many of the above tests are currently popular and are frequently utilized, particularly the light and dark bottle methods, cell enumeration, and chlorophyll concentration. In addition, many of these tests have been used to study the productivity of Lake Livingston.

The most extensive and comprehensive study of phytoplankton productivity in Lake Livingston was conducted by McCullough (1976), in which ^{14}C primary productivity measurements were made at 10 stations within the reservoir 11 times throughout the 7-month study period. The mean productivity for the reservoir during the period of study was determined to be 70.9 $mgC/m^3/hr$ ($n = 211$). The maximum production rate recorded was 358.6 $mgC/m^3/hr$ in the upper portion of the reservoir during the month of July. The minimum production rate reported was 0.8 $mgC/m^3/hr$, also in the upper portion of the reservoir in May. Fig. 2 presents mean primary productivity values for the stations sampled.

It was concluded in the study that the upper portion of the reservoir (north of US 190) and the lower portion of the reservoir (south of US 190) had virtually identical rates of primary production, but were influenced by somewhat different factors. It was determined from multiple regression analyses that the factors most affecting productivity in the upper reservoir were turbidity, phytoplankton standing crop, and incident light. These latter 2 factors were also the major influencing factors in the lower portion of the reservoir, but turbidity was not. The upper portion of Lake Livingston revealed strong negative correlations between phytoplankton productivity and ammonia and nitrate concentrations, but no correlation was found for phosphate, and no correlation was found between productivity and any nutrient parameter in the lower reservoir. (It should be noted that a strong correlation does not necessarily indicate a cause-and-effect relationship, and many factors confound analysis of phytoplankton-nutrient interactions, especially in a non-steady state ecosystem such as Lake Livingston. Algal depletion of nutrients may be offset by the high level of input provided by the Trinity River.)

McCullough (1976) also reported a mean chlorophyll *a* concentration of 24.6 $\mu g/l$ ($n = 109$) for Lake Livingston. A maximum chlorophyll *a* concentration of 69.4 $\mu g/l$ was recorded on August 30, 1975 in the upper portion of the lake. In addition, a highly significant ($\alpha = 0.001$) correlation was reported between chlorophyll *a* concentration and primary productivity ($r = +0.92$).

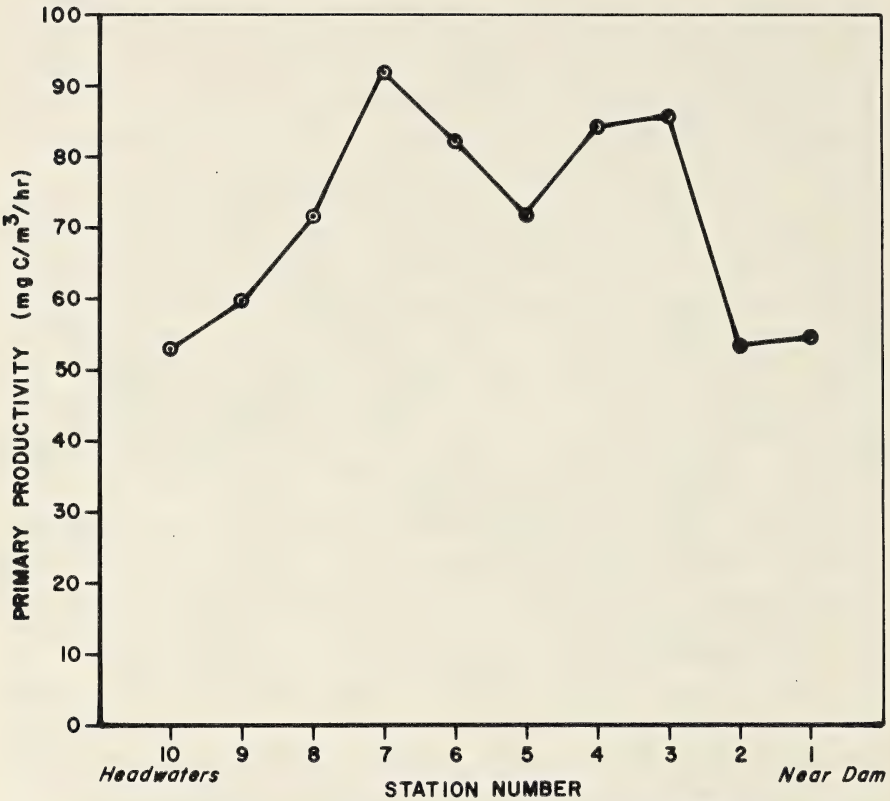

Figure 2. Average phytoplankton primary productivity rates at collecting stations in Lake Livingston from April 6, 1975 to October 25, 1975 (from McCullough, 1976).

TRA (1973-1977) has reported chlorophyll *a* concentrations of 110 μg/l on 2 occasions, both at shallow stations in the lower portion of the reservoir during early June. The highest mean annual chlorophyll *a* concentrations reported by TRA, 42.2 μg/l (n = 7) in 1975 and 33.8 μg/l (n = 8) in 1976, occurred at 2 stations about 6 and 11 mi above US 190, respectively (see Fig. 3).

TWQB (1974-1977) reported a mean chlorophyll *a* concentration of 24 μg/l for Lake Livingston for the period 1974 to date. Summertime mean concentrations for this same time period varied from 32 μg/l (near the dam) to 44 μg/l (at SH 19). Wintertime mean concentrations ranged from 8.3 μg/l (at SH 19) to 11 μg/l (at US 190). The maximum chlorophyll *a* concentration reported by TWQB (1974-1977) was 88 μg/l, which occurred at SH 19 on April 13, 1975. TWQB (1974) reported a maximum chlorophyll *a* concentration of 16 μg/l for an intensive water quality survey conducted during the period November 5-9, 1973, in which 34 stations were sampled.

EPA (1977) conducted both phytoplankton enumerations and chlorophyll *a* analyses in Lake Livingston at 8 stations on 4 occasions in 1974. The results of the phytoplankton analyses are presented in Table 1. It may be seen that the cell

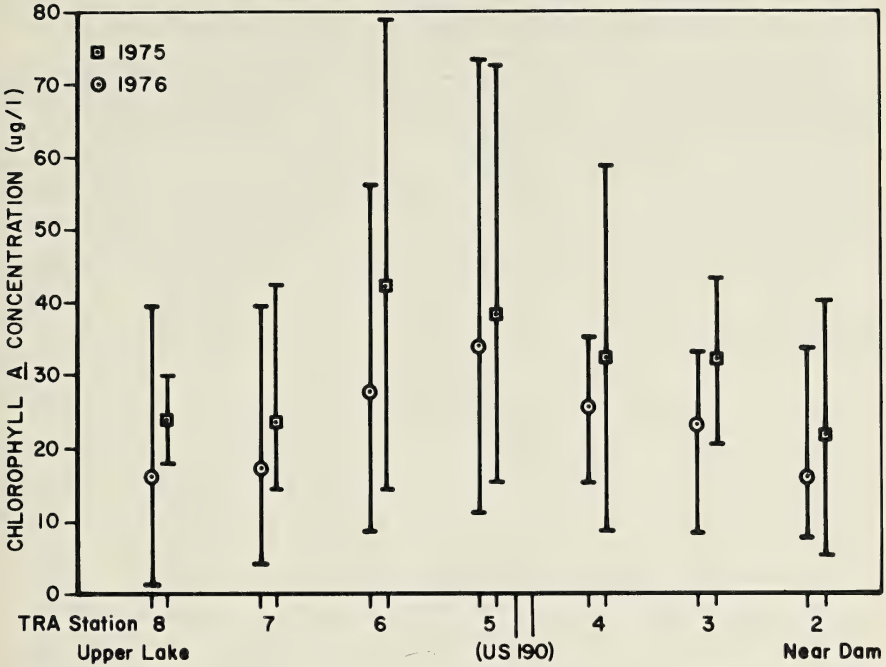

Figure 3. Chlorophyll *a* values in Lake Livingston. Concentrations plotted are means and ranges (data from TRA, 1977c). Stations are mainstem reservoir stations.

counts ranged from over 10^6 cells/l in November to over 15 x 10^6 cells/l in May. These counts are indicative of a high level of primary productivity. The highest mean chlorophyll *a* concentration reported by EPA (1977), 24.1 μg/l (n = 8, s = 2.8), occurred on August 19, 1974 and corresponded to the maximum phytoplankton cell count (see Table 1). Mean chlorophyll *a* concentrations for the March, May, and November samplings were, respectively, 19.4 μg/l (n = 8, s = 13.4), 10.6 μg/l (n = 8, s = 13), and 10.3 (n = 8, s = 5.1). EPA (1977) ranked Lake Livingston the most eutrophic reservoir out of the 39 Texas reservoirs sampled on the basis of 6 physical and chemical parameters measured.

Hydroscience (1976), in the development of a phytoplankton-based eutrophication model of Lake Livingston, utilized chlorophyll *a* data from 4 TWQB surveys of the lake to estimate algal concentrations and productivity. A significant ($\alpha = 0.01$) correlation (r = 0.769, n = 45) was found to exist between chlorophyll *a* and total cell count, with the relation between the 2 expressed by the following equation:

$$\text{Chlor } a \text{ } (\mu g/l) = 0.00835 \text{ Total Cell Count (org./ml)}$$

A recently completed study directed by Dr. McCullough at Stephen F. Austin State University, dealing with primary and secondary productivity measurements in the lake, is presently being finalized. This study is the first to ever measure secondary productivity in Lake Livingston and should provide additional insight into the magnitude of the overall productivity in the lake.

TABLE 1
Phytoplankton Enumeration and Identification for Lake Livingston, Texas.
(Taken from EPA, 1977)

Sampling Date	Dominant Genera		Algal Units/ml
03/16/74	1. *Cyclotella* sp.		5,017
	2. *Melosira* sp.		1,636
	3. *Stephanodiscus* sp.		1,236
	4. *Chroomonas* sp.		763
	5. *Synedra* sp.		473
	Other genera		363
		Total	9,488
05/24/74	1. Centric diatoms		9,691
	2. *Merismopedia* sp.		1,068
	3. *Carteria* sp.		916
	4. *Chroomonas* sp.		687
	5. *Melosira* sp.		686
	Other genera		2,443
		Total	15,491
08/19/74	1. *Dactylococcopsis* sp.		7,832
	2. *Cyclotella* sp.		6,633
	3. *Merismopedia* sp.		6,562
	4. *Oscillatoria* sp.		5,151
	5. *Raphidiopsis* sp.		3,669
	Other genera		2,752
		Total	32,599
11/06-07/74	1. *Stephanodiscus* sp.		521
	2. *Melosira* sp.		137
	3. *Cryptomonas* sp.		137
	4. *Oscillatoria* sp.		82
	5. *Dactylococcopsis* sp.		82
	Other genera		138
		Total	1,097

STUDIES OF LIMITING FACTORS

Organismic requirements for essential materials and conditions are governed by 2 "laws." The first, Liebig's Law of the Minimum, states that the growth of a plant is dependent upon the required nutrient which is present in a quantity near its critical minimum. While this principle was originally applied solely to nutrients, many authors have expanded it to cover other factors, such as temperature, time, and light. Technically, the law applies to steady state conditions only, and any extension to non-steady state systems should be done with caution.

The second "law" governing the concept of limiting factors is Shelford's Law of Tolerance, which states that the tolerance of organisms to one or more factors will limit a population. In essence this is saying that an excess of one factor may be as limiting to a population as an insufficient amount of another.

The limiting nutrient concept is very complex, particularly from an ecosystem dynamics standpoint. Lane and Levins (1977) elaborated on many of the pitfalls

associated with limiting factor experiments. Quite often it is assumed that a given body of water has a particular limiting nutrient, when in fact some non-nutrient factor, which may change regularly from season to season or irregularly due to changes in the weather conditions, may be limiting.

Oversimplification of experimental results or inferences drawn from too few data may lead to erroneous conclusions. Also, the assumption of steady-state conditions within the system being analyzed may be unjustified (this is particularly true for eutrophic bodies of water). Lee (1977) has pointed out that levels of nitrogen and phosphorus considered limiting in northern lakes are not applicable in the south. To compound the problem, phenomena such as factor interaction (the effect of the abundance of nonlimiting factors upon the rate of utilization of the "limiting" factor) and factor compensation (adaptabilitiy to local conditions) exist.

In general, present nutrient limitation experiments are variations of the nutrient spike method, with nitrogen, phosphorus, carbon, and/or micronutrients added. Some experimenters utilize existing phytoplankton communities and some utilize monocultures (e.g., *Selenastrum capricornutum*). Subsequent incubation may take place *in situ* or in the laboratory. Algal response may be detected through the measurement of changes in number, biomass, chlorophyll concentration, etc. (generally the same procedures as used for measuring productivity). After the nutrient spike has been made, changes in the phytoplankton are generally allowed to occur over a period of a few days to several weeks.

Although many micronutrients may limit algal growth and production (e.g., see Goldman, 1972), most of the work related to limiting nutrients has been performed with carbon, nitrogen, and phosphorus. While several limiting nutrient studies have been conducted in Lake Livingston over the past few years, none have been as comprehensive as that of Huang, *et al.*, (1973). The authors used the natural phytoplankton community and determined algal response to additions of nitrogen, phosphorus, and various micronutrients by measuring the change in the optical density (OD) of the samples. All samples were collected in the deepest portion of the reservoir, approximately 500 ft (150 m) from the release tower on the dam. Sampling took place in March, July, and September 1969 and July 1970. Their experiments indicated a somewhat greater response due to the addition of N alone than the addition of P alone. The response from the addition of N ranged from 125-500% of the optical density of the control, whereas the response resulting from P addition ranged from 100-110% of the control. The greatest optical density occurred when N, P, and micronutrients were added together. In the September samples, a substantial increase in optical density was observed only when both N and P were added (OD_{N+P} = 560% of control). Addition of N alone to the sample produced an OD of 179% of the control, but P alone produced an OD of just 85% of the control.

The conclusion reached by Huang, *et al.*, (1973) was that N limited algal growth and production during the summer months (in the main body of the reservoir). However, during late summer, blue-green algae became dominant and seemed to be regulated by the available P.

TWQB (1974) conducted limiting nutrient experiments at 6 stations within Lake Livingston during November 1973 in which algal monocultures of *Selenastrum capricornutum* and *Anabaena flos-aquae* were used. Additions of 0.5 mg/l each of N, P, and N + P were made. The samples were filtered prior to the addition of nutrients and algae and allowed to incubate in the laboratory for 21 days. Dry weight determinations were then made. All stations showed an increase in dry weight ranging from 150-400% of the control resulting from the addition of N alone for the test organism *S. capricornutum.* The addition of P appeared to stimulate the growth of *A. flos-aquae,* although the greatest growth resulted from the addition of N + P. Forty percent of the test results were not reported, however, with only a slight textual reference to "apparent inhibition" resulting from P addition. It may be surmised that the results differed from what was anticipated and, as a result, were not presented.

EPA (1977) conducted studies in March and November 1974 similar to that of TWQB (1974), but differed in that only *S. capricornutum* was used as a test organism and additions of N and P were, respectively, 1.0 mg/l and 0.05 mg/l. The results of these studies are presented in Table 2. It may be noticed that the

TABLE 2
Results of Limiting Nutrient Experiments in Lake Livingston.
(Taken from EPA, 1977)

Spike (mg/l)	Ortho P Conc. (mg/l)	Inorganic N Conc. (mg/l)	Maximum Yield (mg/l-dry wt.)
MARCH SAMPLE			
	Lower Portion of Reservoir		
Control	0.125	0.720	15.4
0.050 P	0.175	0.720	15.1
1.0 N	0.125	1.720	34.7
0.050 P + 1.0 N	0.175	1.720	31.1
	Upper Portion of Reservoir		
Control	0.295	1.014	23.0
0.050 P	0.345	1.014	23.6
1.0 N	0.295	2.014	37.3
0.050 P + 1.0 N	0.345	2.014	41.5
NOVEMBER SAMPLE			
	Lower Portion of Reservoir		
Control	0.096	0.273	1.2
0.050 P	0.146	0.273	1.5
1.0 N	0.096	1.273	3.2
0.050 P + 1.0 N	0.146	1.273	5.5
	Upper Portion of Reservoir		
Control	0.246	0.690	23.0
0.050 P	0.296	0.690	24.0
1.0 N	0.246	1.690	53.9
0.050 P + 1.0 N	0.296	1.690	55.8

greatest increases in dry weight resulted from the addition of N or N + P, but the addition of P produced biomass measurements similar to the control. EPA (1977) concluded that Lake Livingston appeared to be limited by N during the times sampled. However, EPA (1977) pointed out that apparent nitrogen limitation does not necessarily imply that an improvement in a lake's trophic status may be achieved through the reduction of N inputs to the lake.

While presently available data indicate that N is the limiting nutrient in Lake Livingston, primarily during the summer months and particularly in the main body of the reservoir, future studies might want to focus on seasonal differences as well as examine phytoplankton responses from different areas within the reservoir. Studies of factor interactions, factors other than nutrients, and differences between the responses exhibited by monocultures and *in situ* populations might provide further insight into phytoplankton dynamics in Lake Livingston.

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THE IMPACT OF THE O.T.S. ON THE ECOLOGY OF COSTA RICA[1]

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ABSTRACT

This article relates to a visit, Feb. 21–Mar. 7, 1976, made to 3 field stations of the Organization for Tropical Studies in Costa Rica.

INTRODUCTION

Twelve avid bird watchers on a Nature Tour (Annette Cretien, director) joined Dr. Alexander F. Skutch, leader, in San Jose to observe the birds, in particular, and the various aspects of nature, in general, in Costa Rica. A small bus and a native Costa Rican driver made it possible for the group to visit (1) F.S. Palo Verde in Guanacaste, Monteverde cloud forest, El Quizarrá (home of the Skutches), (2) F.S. Las Cruces and Botanical Gardens, Poas Volcano, and (3) F.S. La Selva. At the close of each day the group assembled and under the leader's direction, the birds observed were tabulated as shown on the "log" included in this report.

Costa Rica, although the second smallest republic in Central America with an area of only 19,238 sq mi (Hammond, n.d.), in a biological sense is one of the most richly endowed tropical countries in the world. No point in the country is more than 62 mi from the Atlantic or Pacific Ocean. Within the Republic's 260-mi length, 2 cordilleras follow the long axis, broken only by a plateau known as the Meseta Central. The northern ranges contain a number of volcanic peaks, both active and inactive. Volcano Irazú, with an elevation of 11,260 ft, overlooks the cities of San Jose, Cartago and Turrialba.

The Costa Rican isthmus is geologically young: it arose in the late Miocene and Pliocene periods from volcanic islands which grew and coalesced.

With this preamble concluded, let us set forth on the proposed expedition to the three O.T.S. Field Stations and adjacent points of unique interest as shown on the map. After a visit of 2 or 3 days to a Field Station, we returned to our hotel in San Jose for a day and a night.

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[1]Presented at Annual T.A.S. session, Baylor Univ., Waco, Texas, March 11, 1977.

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This chart serves as a log of the different kinds of birds observed en route to and at the stations visited on a 13-day nature tour.

STATIONS:	San Jose	Palo Verde	Palo Verde	Monte Verde (Earthquake)	Monte Verde	San Jose (Free Day)	San Jose	El Quizarrá	Las Cruces	Las Cruces (Quetzal)	Poas	La Selva	La Selva
BIRDS: DAY:	1	2	3	4	5	6	7	8	9	10	11	12	13
Grebes, Cormorants, Anhingas, Herons, Bitterns	4	7	7	0	1	1	0	2	0	1	3	0	0
Storks, Ibises, Spoonbills, Ducks, Amer. Vultures, Hawks, Kites, Eagles	14	13	10	2	4	0	0	3	3	3	3	3	5
Ospreys, Falcons, Caracaras, Guan, Limkins, Rails, Gallinules, Coots	4	2	3	0	0	0	0	0	0	0	1	0	0
Sungrebes, Jacanas, Sandpipers, Snipes, Stilts	1	4	3	0	1	0	0	0	0	1	2	0	0
Thick Knees, Pigeons, Doves, Parrots, Parakeets	6	6	10	2	4	3	2	2	2	2	4	2	4
Cuckoos, Anis, Nightjars, Swifts	2	2	1	2	1	2	0	1	2	1	2	1	1
Hummingbirds, Trogons	1	0	3	2	1	1	1	7	4	4	1	4	3
Kingfishers, Motmots, Puffbirds, Toucans, Woodpeckers	2	2	2	2	4	1	0	2	3	2	5	5	4
Woodcreepers, Horneros, Spinetails, Antbirds	0	1	0	2	0	0	0	3	7	2	3	5	6
Manakins, Cotingas	0	1	2	1	0	0	0	2	3	0	1	3	3
Tyrant-Flycatchers	2	5	5	4	2	3	0	8	8	6	5	5	5
Swallows, Jays, Wrens	5	6	5	3	4	3	0	7	8	6	4	2	2
Thrushes, Gnatcatchers, Silky-Flycatchers, Strikes, Vireos, Honeycreepers	2	4	2	1	0	6	0	4	5	3	4	3	3
Wood Warblers, Amer. Orioles, Blackbirds	1	3	4	4	2	6	0	7	9	2	4	4	5
Tanagers	5	4	2	3	0	3	0	9	11	4	3	7	8
Finches	2	2	1	2	2	2	0	6	4	2	3	5	7
Total number of different kinds of birds recorded each day	51	62	61	30	26	31	3	63	69	39	48	49	56

Palo Verde F.S.

The Palo Verde Field Station, in the Guanacaste Province, is situated 25 mi south of the Pan-American Highway and was our first major stop. The O.T.S. building is at the foot of the limestone Palo Verde Hills overlooking the flood plain of the Rio Tempisque. Trees in this "dry forest" vary in height from 65 to 70 ft with crowns of small leaves or leaflets. A thick understory of smaller trees,

NICARAGUA
83°N
11°W
11°E
83°S
Tortuguero
CARIBBEAN SEA
3 La Selva Field Station
1 Palo Verde Field Station
Irazu Volcan
SANJOSE
TURRIALBA
CARTAGO
Islas de Cuyabo et Negritos
PACIFIC OCEAN
PANAMA
2 Las Cruces Field Station
Pan American Highway
FIELD STATIONS AND ECOLOGY OF COSTA RICA
ECOLOGICAL ZONES
Tropical Dry Forest and transition - lowland and premontane
Tropical Moist Forest and transition - lowland, premontane and lower montane
Tropical Wet Forest and transition - lowland, premontane, lower montane and montane
Rain Forest - premontane, lower montane and montane
Subalpine Rain Paramo
Adapted from Republica de Costa Rica MAPA ECOLOGICO by Joseph A. Tosi jr
Zones based on precipitation, evapotranspiration potential, temperature, and elevation.

some of them evergreen, in addition to vines and low branched shrubs, gives the forest a tangled appearance. Occasional very large specimens of *Enterolobium cyclocarpum* (Guanacaste tree, the national tree of Costa Rica, for which the province is named) are present.

Ceiba pentandra (silk-cotton tree) and *Samanea saman* (rain tree), sometimes 40 m high, stand out above the other trees; trunks are often buttressed. Natives use the trunks, because the wood is easily worked, for canoes, split posts, matches, packing boxes, platters, furniture, and wheels cut from cross-sections of the buttresses. In addition, an important product of the first named is the silky fiber enveloping the seeds which is very fine, light, elastic and does not become matted under pressure. It is sold under the name "kapok" and is used for stuffing mattresses, pillows, life preservers, and in England to make beaver hats. The last named tree is planted in Costa Rica as a cacao shade. When they fall, the flowers of both trees and the seed pods of the Saman are eaten by stock (Standley, n.d.). Hundreds of flowering trees studded the plain. One locally called corteza or yellow poui (*Tabebuia chrysantha*), wore brilliant yellow blossoms. Later as we drove farther north, the yellow was supplanted by the soft pink of a similar tree, pink

poui (*Tabebuia rosea*) (Pertchik, 1951). Since 1963, grazing land in Costa Rica has been doubled; once a tropical forest is cut down, it is gone forever. Mute evidence of this loss is observed in the numbers of large tree trunks hauled out on trucks.

On the lowlands, subject to seasonal floods, there are almost pure thickets of the spiny palm and the Palo Verde (*Parkinsonia aculeata*) from which the Field Station takes its name.

Directly in front of the O.T.S. building site, the marshy flood plain of the Rio Tempisque is more than 2 mi wide. In this area large numbers of aquatic birds were present: (Least and Pied-billed) grebes, cormorants, anhingas, (Great blue, Green and Little blue) herons, (Great, Snowy and Cattle) egrets, (Wood and Jabiru) storks, ibises, spoonbills, (Black-bellied Tree, Blue-winged Teal and Northern Shoveler) ducks, (King, Black and Turkey) vultures, falcons, caracaras, limkins, coots, jacanas and stilts. This was an experience beyond compare.

The golden toad, which is endemic to the Monte Verde cloud forest, regretfully hid while we were present; whereas iguanas, male, female and offspring were numerous and basked in the sun on fallen tree trunks in the Palo Verde marsh.

Among the unusual mammal species found were monkeys, sloths, coyote, porcupine, agouti (guinea pig family, size of rabbit), and coati (allied to raccoon). Caretakers at the Field Stations tame members of the last 2 named.

Of the 200 and more different species of birds observed and tabulated by our group, by far the most glamorous was the resplendent quetzal, national bird and emblem on the coin of Guatemala. Since the leader had made a special study of and published on this rare bird, the group was confident that they would see it. As a consequence, the members sat for 3 hrs in the cattle pen of a hacienda watching, but with no luck. Even though the quetzal evaded us, the visit to the Monte Verde cloud forest provided excitement: an earthquake of momentary duration occurred.

Las Cruces F.S.

On the journey to Las Cruces F.S. the group made a brief stop at El Quizarrá, the Skutch plantation, near San Isidro. While there, the leader halved a grapefruit and half peeled a banana and placed these on a bird feeder. Quickly the birds responded. In a twinkle, a hummer descended and partook of the citrus and several species of honey creepers partook of the banana while brilliantly colored tanagers darted about. The greatest number of hummers (Greenwalt, 1960; Skutch, 1973) were found here. See chart, Day 8.

Vegetation at the Las Cruces Field Station is classified as a Premontane Rain Forest, while a cloud forest occupies the upper ridges adjacent to the station. Trees of the forest are chiefly broad-leafed evergreens which form a canopy at about 98 ft and an exquisite carpet under foot when the leaves fall. A preliminary list of trees constituting the canopy and understory includes about 80 species

(Organization for Tropical Studies, 1972). Tree ferns are particularly well represented and lower strata of the forest are rich in epiphytic and terrestrial ferns.

In addition to the natural forest, the Las Cruces F.S. contains garden collections of epiphytes, palms, cycads, tree ferns and other trees and shrubs, imported from both old and new world tropics. These plantings are continually being enlarged. The Las Cruces site includes a botanical garden of about 50 acres developed by Mr. and Mrs. Robert Wilson.

Members of the Orchidaceae under cultivation in the garden number over 1000 species and hybrids. Well over 100 species are native to Costa Rica. Tree ferns grow luxuriantly in the area: planted along the contoured walks are more than 500 specimens, representing 25 species, 12 species native to the area. More than 350 species of *Bromeliaceae* are planted over the Stanley Smith Science Laboratory. In the banana family, *Heliconia* is a large genus which bears colorful flowers. The garden includes 19 of the 25 species native to Costa Rica. Also with showy flowers are the gingers.

Ground cover species are particularly important because of steep slopes and high rainfall. Especially successful is *Ophiopogon japonicum,* Japanese turf lily, whose intricate and extensive root system will hold a nearly vertical surface intact against erosion. Five other genera, including *Selaginella* are being tested.

Each acquisition brought into the garden is given a number and at present they total more than 4,000. Complete records and a color slide are maintained by accession book and card file.

On our return journey from the Las Cruces F.S. to San Jose, the leader again received word that a quetzal had been sighted. We watched patiently for an hour before a second false alarm was declared. Pamela Skutch, wife of the leader, was about to reenter the bus when fate shifted and she sighted the vagrant. We could hardly believe what we saw! (cf. Wheeler, 1967; Line, 1975).

La Selva F.S.

Only 2 hrs or 40 mi from San Jose en route to La Selva Field Station is the active volcano, Poas, surrounded by a cloud forest. It is Costa Rica's most visited and best developed National Park. Its elevation ranges from 5,300 to almost 9000 ft. The main features are the active crater and an extinct volcanic cone with a small fresh water lake inside.

Hummingbirds are Poas' most characteristic animal. The endemic Poas Mountain squirrel and armadillo are mammals commonly seen.

Orchids, bromiliads and other epiphytes are the stars of Poas' floral display, often breaking large branches off of dead trees by their combined weight and numbers. Ferns, mosses, lichens and fungi use these fallen trunks as "nurse trees."

The La Selva F.S. is situated in an undisturbed rain forest at the confluence of 2 rivers. Movement through the forest is generally possible because low light intensity results in a sparse undergrowth. "Epiphytic growth on trees is abundant at all levels. At least 200 species of trees are present at La Selva, the commonest

being *Pentaelethra macroloba,* a legume which comprises about 30% of the basal area" (Organization for Tropical Studies, 1972).

Two arboreta being developed at La Selva contain over 700 trees with more than 100 species labeled.

The Palo Verde F.S. area on the Pacific side which contains typical examples of the Tropical *Dry Forest* is under investigation in the O.T.S. Ecosystem Comparison Study with the Tropical *Wet Forest* of the La Selva F.S. on the Atlantic side of Costa Rica. Within these 2 areas a wide variety of habitats offers an excellent opportunity for study of both natural communities and those which result from the impact of agricultural usage.

For these study areas, a grid system has been laid out with permanent stakes set at 200 m intervals. Trails are maintained to enable investigators to enter particular areas with least disturbance to the vegetation. At strategic locations, shelters provide protection from sudden rain and a place for equipment.

Recorded on magnetic tape, information is processed by a computer in the O.T.S. Office in San Jose and stored on punched tape and magnetic discs. Instruments are available to moniter soil conditions, such as flow of water, movement of minerals, conductivity, pH, and temperature. Dendrometers automatically record changes in the diameter of selected trees.

An estimated 350 species of birds are residents or visitors at some time during the year (Organization for Tropical Studies, 1972). One-half of the bird species of the world inhabit rain forests.

Access to the La Selva F.S. is usually by surface transportation to the village of Puerto Viejo, thence by small boat to the Field Station.

CONCLUSION

Each day of the Nature Tour 30 to 60 species of birds were tabulated, the greatest number, as shown on the chart, were seen at the 3 O.T.S. Field Stations.

Buses are practically the only vehicles encountered on the sinuous Pan American Highway except for the immense trucks used to haul the giant trunks of trees, unfortunately, cut to clear the land for herds, chiefly of Brahman cattle.

The contemplative life prevails in Costa Rica with relatively few very poor or very rich whereas the active and experimental prevails in the Field Stations. The ecologically different areas in Costa Rica chosen for the 3 O.T.S. Field Stations offer a wide variety of habitats for study, it is true, but has the O.T.S. been able to make its influence felt? Yes, subtly.

ACKNOWLEDGEMENTS

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DOSTOEVSKI AND OPARIN: THE FATE OF MAN

An essay regarding the effects of philosophy on the nature of man

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INTRODUCTION

It now seems that the mechanistic interpretation of man is destined to triumph. Although this is in some ways a melancholy prospect, yet in others it manifests surprising possibilities for new modes of human satisfaction. We who are living through the present debate over the future of man know that it is partly a time of loss, a time of painful re-examination and of constant looking into mirrors and even behind the mirrors. We believe that Fyodor Dosteovski, the moralist, and A.I. Oparin, the scientist, have emerged as important symbols in the debate. Few thinkers so clearly dramatize the issues of the past hundred years as these 2 Russian giants.

Dostoevski stands for all men who wish to believe that man has the capacity to exercise free judgments and hence to be held morally accountable for his actions. Oparin stands for those who believe that man is the necessary consequence of molecular evolution and that he is not a free anomaly in nature. Their separate truths thus seem, *prima facie*, irreconcilable. Indeed, they appear to constitute some keen dividing wedge which splits the truth into 2 parts, forcing all men to choose: Dostoevsky (man as moral being) or Oparin (man as the synthesis of natural processes).

In Oparin's elegant theory of the origin of life on earth, man is the natural product of a series of chemical reactions of increasing complexity determined only by the nature of the chemical elements involved and the conditions under which the reactions occur. This implies that man, including his mental life (and the very thoughts of Oparin himself), is reducible to chemical equations. Love,

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hate, compassion and greed are all the results of simple reaction kinetics. Freedom and will are but the remote fantasies of a deluded, humanistic past which is also the synthesis of other reaction kinetics. On the other hand, in Dostoevsky's universe, moral responsibility, guilt and righteousness would be vitiated should the materialistic view of man prevail. In this view, man is spiritual and his salvation is possible only through contrition and suffering. Does this triumph of Oparin mean that man is doomed? Are we facing a Dostoevskian hell? Is there to be an Oparinian salvation? It is our judgment that both Oparin and Dostoevsky tell the grim truth, and together, perhaps, they make us face an unaccustomed world in which man is seen as both chemical and moral.

But it is a tenable world and this is our thesis.

The processes that Oparin described make it clear that in terms of modern understanding, the "secret of life" is becoming daily a more open secret and that it is a chemical secret. The philosopher's lodestone was not necessary and in fact, it was not even a very dramatic secret. The chemistry of DNA, proteins, and enzymes locked up in a fantastic multitude of chemical reactions and equilibria is not as esoteric and complex as once thought. Today the deciphering of this secret is a commonplace routine even in very modest laboratories throughout the world. Man long expected this secret to abide as the last proud bastion against rational inquiry, but the wall has been breached in a hundred humdrum places not by Merlin or Mohamed, or even Oparin, but by the scores of unassuming, journeyman chemists who have followed him.

Yet such knowledge is not sufficient, finally, to vitiate Dostoevski's moral vision of the world. Indeed, it is possible that the future may require of us that we reconcile the best lessons of Dostoevski with the best in Oparin, that man learn to respect his deepest feelings and his capacity for moral conduct precisely because he is a bio-chemical complex whose survival is contingent not on a transcendental absolute, but (within the limits of nature) on his own ingenuity and desire to survive. For the force of our feelings is quite enough to generate the troubling conflict between our wish to be good men and the necessity of compromising our ideals. Suppose the last chemical mechanism were known and could be formulated, the lesson of Oparin; if that reaction constituted the feeling of love, the knowledge would justify itself, but it would not replace love, the lesson of Dostoevski. The knowledge of man's materialistic nature need be disconcerting only if we believe that mysteries are intrinsically richer and more interesting than things known, or if we naively assume that the knowledge explosion actually threatens to exhaust the supply of mysteries in this amazing and complex universe. A dramatic shift in our emotional responses to things known is thus in order if we are to be at home in Oparin's world. And few writers of any time have known more about the feelings of man than Fyodor Dostoevski. Indeed, Dostoevski, (who would be less reconciled to Oparin than Dostoevski himself) has shown us a way to live by rehabilitating and emphasizing the role of feelings in modern man. Few have so keenly seen that such secrets as Oparin

would reveal are the work, not of a rational species, but of a species in whom the rational and irrational are so wildly mixed that one can only wonder at the modest victories of the rational. Those who feel with Keats that "Philosophy will clip an angel's wings," who mourn the loss of an enchanted past, merely forfeit the possibility of an enchanting future. It seems mere melodramatic posturing to suppose that a rainbow, understood as the prismatic separation of light by droplets of water is intrinsically less wonderful than the rainbow as a promise of God. Similarly, it tends to be but a spurious humanism which laments the triumph of a rational account of man's bio-chemical history. Our dilemma is not that we know too much, but that we do not wonder at what we know, that we wonder too little. Oparin, and perhaps, Darwin too if they closed an era in the history of our ideas about life on this planet, also opened an era. The spiritual masochism of modern times has merely sapped our ability to wonder at it, yet, ironically, even that despair may yet become a resource of strength. When the old peasant woman comes to see Dostoevski's Father Zossima, seeking comfort for the death of her only child, the old priest tells her that no comfort is possible, that her sorrow is greater than the sorrow of all other women and she walks away in grief. Incredulous, Alyosha Karamazov asks the old priest why he has offered her no solace, but Father Zossima replies that it is her great grief which sets her apart from others, it is the secret and the strength of her life. And so for man, the child is dead, but the sorrow and desperation of it all may yet yield a depth and richness in the coming age which will be tantamount to the redemption of those who come to feel it and understand it. A scientific age need not be mechanistic or superficial; its truths need not all be sterile explanations. If we should lose the grace of being human, it will be by default - not because we have lost our immortality, but because we haven't stopped whining about it, not because our interests became scientific, but because we tried to exorcise the non-rational element of our sensibilities and so found ourselves at war with our own deepest instincts. We, in the twentieth century, are the victims of this conflict and not of science or of lost innocence.

Hopefully, science has come of age. It no longer needs the absolutist air of a century ago to bolster up a boyish pride. It can accept the joy of its successes, the sorrow of its failures, and the challenge of its ignorance without special pleading and with a dignity and tranquility which transcends polemics. Let scientism (as opposed to science) die; let humanism (as opposed to the human) die. Their polemical quarrel has become both tedious and irrelevant. Let man be. Ignorant and anxious, proud and full of sympathy, turbulent and half-mad, whatever he is, let him be. Perhaps Omar Khayyam foresaw such an option:

> And if the Cup you drink, the Lip you press,
> End in what All begins and ends in - Yes;
> Imagine then you *are* what heretofore you *were* -
> Hereafter you shall not be less.

Thus to accept man as he is, not as we would have him be, embodies a paradox, for in accepting him we accept not a static entity, but a dynamic and evolving

entity -- this is the mutual "less" of Dostoevsky and Oparin. The thing we accepted today will be a different thing tomorrow; and that is a mystery in itself which we must accept in order to avoid spiritual masochism and philosophical irrelevancies. To affirm the social or political *status quo* is, ironically, not to accept man at all; it is to deny human vitality, the fundamental fact of man's being. Here, perhaps, Oparin and Dostoevsky unite against the utopians who have appeared among the behavioral psychologists, for both repudiate the stasis which is characteristic of dwellers in the Crystal Palace. The threat of behavioral psychology lies less in the certainty of its errors than in the possibility of its truth. It may put within our power the ability to gain our perverse wishes for man, to make of him what we want him to be rather than to have him as he is, and the danger is that we will see these wishes as perfection. It may be that our wants will be so banal and so venal that we will produce the reality which Dostoevsky most feared and which Oparin's heirs have shown to be such a trivial yardstick by which to measure man.

Notes Section

STONE-EATING AND TOOL-USE AMONG OLIVE BABOONS—While studying a group of olive baboons (*Papio cynocephalus anubis*) in Nairobi National Park, Kenya, on 28 and 29 September 1972, I observed a troop of 37 baboons systematically collect, ingest, and masticate pea-sized stone fragments. The stone-eating sequences, each of which lasted about 20 min, took place in an area where approximately 3 m of overburden had been removed several years previously in order to build a roadway across the mouth of Sosian Gorge, in the south central portion of the Park. The area involved was approximately 400 m down the gorge from a natural salt lick which was commonly visited by the baboon troop.

Although the behavior and diet of olive baboons have been studied extensively (Washburn and DeVore, 1961, *Sci. Amer.*, 204:62; Harding, 1973, *Sci. Amer.*, 204:344), no previous reports include stone fragments in the eclectic diet of this diverse species. A preliminary analysis of rock and soil samples collected in the area--primarily decaying tectonic materials and laterite clay--was non-conclusive, no unusual concentrations of salts or trace elements were observed. Because of the ready availability of seemingly more accessible salts at the nearby lick, unless the stony materials provide trace elements or oral sensations which are otherwise unavailable, one can only speculate as to why baboons would subject themselves to such physical unpleasantry as chewing stones.

In addition to stone-eating, another rare behavior among olive baboons, tool-use was documented. During the 29 September stone-eating session, an adult male baboon was observed for about 3 min as he used a twig as a primitive, though effective, tool. The twig, approximately 5 mm in diameter and 10 cm long, was held in a precision grip (Napier, 1962, *Sci. Amer.*, 207(6):56). It was manipulated in such a way that it enabled the baboon to more effectively extract and separate "bite-size" stone fragments from their rock-and-clay matrix. The tool-use ceased abruptly, and was not repeated, as I moved closer to the baboon in order to better observe and photograph the proceedings.

While the stone-eating and tool-using episodes which have been reported stand as rare and isolated instances--tool use in feral baboons has been reliably reported only once (van Lawick-Goodall, *et al.*, 1973, *Nature* (London) 243:212)--these observations demonstrate that because primatology is still in a data-gathering stage of development, we should perhaps expect the unexpected. If we assume that monkeys, apes, or humans are incapable of certain behaviors, we may not recognize them if they do occur.

I am indebted to Mr. Perez Olindo and the National Park System of Kenya, and to Doctors H.B. Sarles of the University of Minnesota and A.C. Walker of Harvard University for their cooperation, kindness, and support—*Ordean J. Oyen, Anthropology Research Laboratories, Texas A&M University, College Station 77843.*

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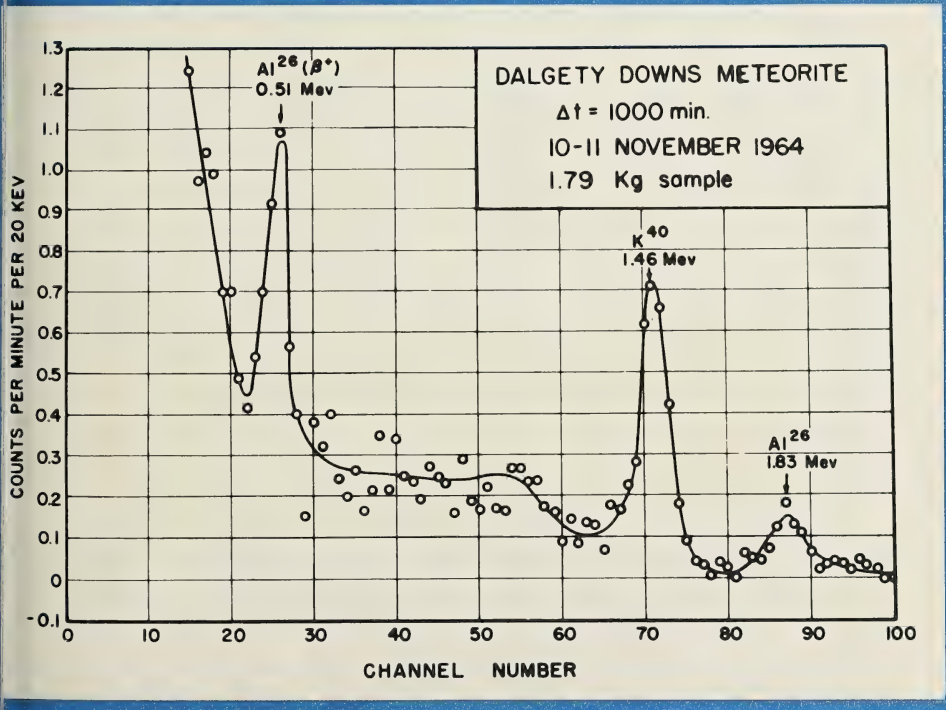

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A TRANSFORMATION ON CORNISH-FISHER EXPANSIONS

by E.D. McCUNE *and* J.E. ADAMS

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ABSTRACT

A general method of computation for approximating the quantiles of a distribution function by a transformation on Cornish-Fisher expansions is described and exemplified. Asymptotic properties are also discussed.

INTRODUCTION

The Cornish-Fisher expansions were first introduced by Cornish and Fisher (1937) and later reformulated by Fisher and Cornish (1960). Interest in the theory as well as the application of the expansions in statistics has been shown in papers by Wasow (1956), Wallace (1958), Finney (1963), Bol'shev (1959, 1963), Hill and Davis (1968), and more recently by Draper and Tierney (1973), McCune and Gray (1975), Shenton and Bowman (1975), and McCune (1977). Many researchers have found direct applications of the Cornish-Fisher expansions in such areas as Genetics (Panse, 1940), Analytical Chemistry (Kelly and Harris, 1971), Dental Research (Dubey, 1966), Computer Science (Hill and Davis, 1973), as well as to many nonparametric techniques being used in the areas of Social Science and Business (Bickel, 1974). The application of these expansions has been hampered because of the difficulty in obtaining the terms of the expansions, the difficulty increasing with the number of terms desired. Although in some cases tables have been produced to alleviate the problem, the difficulty in calculating the higher order cumulants for the increasing number of terms is always present. Hence after k terms of an expansion are obtained, it is of utmost importance to make the most efficient use of these terms.

A similar problem in numerical analysis has been studied under the heading of non-linear transformations of divergent and slowly convergent sequences (Lubkin, 1955; Shanks, 1955; Clark, *et al.*, 1969). In one example (Lubkin, 1955), it has been demonstrated that by using only 9 terms of a series and making the appropriate use of a transformation on the sequence of partial sums more information was obtained than using 10^9 terms of the original series. Results of this

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type even on a much smaller scale would certainly be welcomed by users of the Cornish-Fisher expansion.

In this article a transformation is recommended which should be of value in reducing the number of terms needed when using Cornish-Fisher expansions.

DEFINITIONS AND THEOREMS

Let y and x be corresponding quantiles of distribution functions F and G such that the Cornish-Fisher expansion of y in terms of x exists. It is assumed throughout this paper that, after possibly grouping some terms, the expansion can be displayed in the form

$$y = a_0(x;\lambda) + a_1(x;\lambda) + a_2(x;\lambda) + \ldots \tag{1}$$

where no a_i is the zero function, $a_i(x;\lambda) = 0(\lambda^{\alpha_i})$ as $\lambda \to \infty$ for $i = 1,2,3,\ldots$, and $0 > \alpha_1 > \alpha_2 > \ldots$, with $\alpha_{i-1} + c = \alpha_i$ where c is a negative constant independent of i. nt of i.

Under certain conditions (Bol'shev, 1963; Draper and Tierney, 1973; Wallace, 1958; Wasow, 1956) equation (1) satisfies the asymptotic property

$$y - y_m = 0(\lambda^{\alpha_{m+1}}) \tag{2}$$

as $\lambda \to \infty$ where

$$y_m = \sum_{i=0}^{m} \alpha_i(x;\lambda). \tag{3}$$

Actually equation (3) is referred to in literature as the Cornish-Fisher expansion up to terms of order $0(\lambda^{\alpha_{m+1}})$. The M_m transformation on y_m will now be defined.

Definition 1: For $m \geq 2$,

$$M_m = [(y_m)(y_{m-2}) - (y_{m-1})^2]/(y_m + y_{m-2} - 2y_{m-1})$$

where $y_m + y_{m-2} - 2y_{m-1} \neq 0$.

It might be noted at this point that M_m is simply the T_{m-1} transformation introduced by Lubkin (1955). However the notation M_m is more appropriate in this case since it stresses the point that no more information is needed to calculate M_m than y_m.

Theorem 1: If equation (2) is valid and

$$\lim_{\lambda \to \infty} \lambda^{-\alpha_i} a_i(x;\lambda) = L_i(x)$$

for i = m-1 and m, then

$$y - M_m = 0(\lambda^{\alpha_{m+1}}) \text{ as } \lambda \to \infty.$$

Proof: The proof follows immediately using the identity

$$M_m = y_m + \{[a_m(x;\lambda)]^2 / [a_{m-1}(x;\lambda) - a_m(x;\lambda)]\}.$$

It can be concluded from Theorem 1 that y_m and M_m are asymptotically equivalent as $\lambda \to \infty$; however in statistical applications we are generally concerned with the approximation y_m when λ is finite and quite small. Some examples will now be presented which indicate that M_m can be very valuable for increasing the accuracy of y_m for finite λ.

EXAMPLES

Example 1: Let Φ be the N(0,1) cdf and let F be the standardized gamma cdf, that is

$$F(x;\lambda) = \int_{-\infty}^{x} \lambda^{1/2} g(t\lambda^{1/2} + \lambda)dt, \tag{4}$$

where

$$g(u) = [\Gamma(\lambda)]^{-1} u^{\lambda-1} e^{-u} \quad u > 0$$
$$= 0 \qquad \text{elsewhere.} \tag{5}$$

Let y and x be corresponding quantiles of Φ and F, that is

$$F(x;\lambda) = \Phi(y).$$

The Cornish-Fisher expansion of y in terms of x (Johnson and Kotz, 1970) can be expressed in the form of equation (1) by selecting $a_o(x;\lambda) = x$ and for $i \geq 1$, select $a_i(x;\lambda)$ such that $\alpha_i = -(i/2)$ with $c = -1/2$. Hence,

$$a_1(x;\lambda) = (1-x^2) / (6\beta_3),$$
$$a_2(x;\lambda) = (3x-x^3) / (24\beta_4) + (4x^3-7x) / (36\beta_3^2),$$
$$a_3(x;\lambda) = (-x^4 + 6x^2-3) / (120\beta_5) + (11x^4-42x^2 + 15) / (144\beta_3\beta_4)$$
$$+ (-69x^4 + 187x^2-52) / (648\beta_3^3)$$

and so on where

$$\beta_i = 0 \qquad , i = 1,2$$
$$= (\lambda^{1-(i/2)}(i-1)!)^{-1} \qquad , i = 3,4\ldots .$$

Both y_m and M_m are easily calculated. We compare the approximations by investigating the accuracy of $\Phi(y_m)$ and $\Phi(M_m)$ in approximating F. The results are displayed in Table 1 and it appears that for finite λ, $\Phi(M_m)$ is uniformly much more accurate than $\Phi(y_m)$.

TABLE 1

λ	x	F	$\Phi(y_3)$	$\Phi(M_3)$	$\Phi(y_4)$	$\Phi(M_4)$
5	1	.84748	.84678	.84739	.84774	.84748
	2	.95902	.95186	.95877	.96356	.95910
	3	.99069	.96919	.99035	.99790	.99087
	4	.99812	.87458	.99788	1.00000	.99828
15	1	.84362	.84353	.84361	.84364	.84362
	2	.96528	.96449	.96524	.96561	.96529
	3	.99442	.99322	.99437	.99505	.99443
	4	.99930	.99835	.99928	.99967	.99931
25	1	.84276	.84272	.84275	.84276	.84276
	2	.96763	.96734	.96761	.96772	.96763
	3	.99552	.99515	.99550	.99568	.99552
	4	.99955	.99935	.99954	.99964	.99955

Example 2: Let y be the chi-square quantile and let x be the corresponding standardized Normal quantile. The Cornish-Fisher expansion of y in terms of x (Fisher and Cornish, 1960) can be expressed in the form of equation (1) by selecting

$$a_o(x;\lambda) = \lambda + (2\lambda)^{1/2}x + (2x^2-2)/3$$

and for $i \geq 1$, select $a_i(x;\lambda)$ such that $\alpha_i = (-i/2)$ with $c = -1/2$. Hence

$$a_1(x;\lambda) = (x^3-7x)/[9(2\lambda)^{1/2}],$$
$$a_2(x;\lambda) = (-6x^4-14x^2+32)/(405\lambda),$$
$$a_3(x;\lambda) = (9x^5+256x^3-433x)/[4860(2^{1/2})(\lambda)^{3/2}]$$

and so on. From Table 2, it can be observed that M_m does a nice job of improving the accuracy of y_m.

Example 3: Let y be the student-t quantile and let x be the corresponding standardized Normal quantile. The Cornish-Fisher expansion of y in terms of x (Fisher and Cornish, 1960) can be expressed in the form of equation (1) by selecting $a_o(x;\lambda) = x$ and for $i \geq 1$, select $a_i(x;\lambda)$ such that $\alpha_i = -i$ with $c = -1$. Hence

$$a_1(x;\lambda) = (x^3+x)/(4\lambda),$$
$$a_2(x;\lambda) = (5x^5+16x^3+3x)/(96\lambda^2),$$
$$a_3(x;\lambda) = (3x^7+19x^5+17x^3-15x)/(384\lambda^3)$$

and so on. A comparison of M_m with y_m is displayed in Table 3 and again we find that M_m provides an increase in accuracy.

TABLE 2
Approximations of Chi-Square Quantiles

	λ	.950	.990	.999
EXACT VALUE	3	7.8147	11.3449	16.266
y_3		7.8197	11.3706	16.338
M_3		7.8156	11.3465	16.260
y_4		7.8086	11.3326	16.254
M_4		7.8137	11.3449	16.276
EXACT VALUE	6	12.5916	16.8119	22.458
y_3		12.5932	16.8192	22.477
M_3		12.5921	16.8125	22.456
y_4		12.5904	16.8097	22.456
M_4		12.5914	16.8121	22.460
EXACT VALUE	20	31.4104	37.5662	45.315
y_3		31.4106	37.5670	45.317
M_3		31.4105	37.5663	45.314
y_4		31.4103	37.5661	45.315
M_4		31.4104	37.5663	45.315

TABLE 3
Approximations of Student-t Quantiles

	λ	.9750	.9900	.9990
EXACT VALUE	5	2.571	3.365	5.893
y_3		2.568	3.355	5.797
M_3		2.572	3.371	5.965
y_4		2.570	3.364	5.875
M_4		2.571	3.366	5.903
EXACT VALUE	7	2.365	2.998	4.785
y_3		2.364	2.995	4.762
M_3		2.365	2.999	4.782
y_4		2.365	2.998	4.782
M_4		2.365	2.998	4.787
EXACT VALUE	10	2.228	2.764	4.144
y_3		2.228	2.763	4.138
M_3		2.228	2.764	4.146
y_4		2.228	2.764	4.143
M_4		2.228	2.764	4.144

SUMMARY

It has been shown that y_m and M_m are asymptotically equivalent as $\lambda \to \infty$. For finite λ, which is generally the case in statistical applications, examples have been given for which M_m is a considerable improvement in accuracy over y_m. Further research to determine under what conditions M_m is more accurate than y_m for finite λ would certainly be of interest.

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THERMAL WATER OCCURRENCES IN TRANS-PECOS, TEXAS

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ABSTRACT

Thermal water has a temperature equal to or greater than 30 C (86 F). Forty-four occurrences from springs and wells in a 6-county area of Trans-Pecos, Texas have been located, sampled, and chemically analyzed. Springs account for 19 of the 44 thermal water occurrences. The largest number of thermal water occurrences (14) are found in Presidio County.

The thermal waters can be divided into 2 groups based upon total dissolved solids; in the first group total dissolved solids exceed 5000 ppm and in the second group they are less than 3000 ppm. Thermal waters contain higher concentrations of sodium, potassium, silicon, lithium, fluoride, chloride, bromide, iodide, and manganese and lower concentrations of copper, zinc, lead, and iron than nonthermal waters of the region.

INTRODUCTION AND METHODS

A number of warm-to-hot springs and wells have been reported along both sides of the Rio Grande River in west Texas and northern Mexico, (Groat, 1972, Warning, 1965). However, numerous unreported occurrences of thermal waters occur in the region and very little data are available concerning the chemistry of these waters.

During a current geothermal exploration program in west Texas, an attempt has been made to locate all thermal water occurrences on the United States side of the Rio Grande River (Fig. 1). From June 1976 to August 1977, 1028 water samples were collected from a 22,000 mi^2 area in 6 counties of Trans-Pecos, Texas. For each occurrence, temperature and pH were recorded in the field, then samples were sent to the laboratory for chemical analyses. Water samples were collected from wells and windmills only after allowing water to flow for at least 15 min. Total dissolved solids were obtained by the use of a Kernco DS meter. After collection, each sample was acidified with 1 ml of nitric acid to prevent metals from plating out on the container walls. Because of the addition of nitric acid, tests for bicarbonate and sulfate ions could not be made. Several duplicate samples were analyzed by an independent laboratory and show good agreement with those presented in this report.

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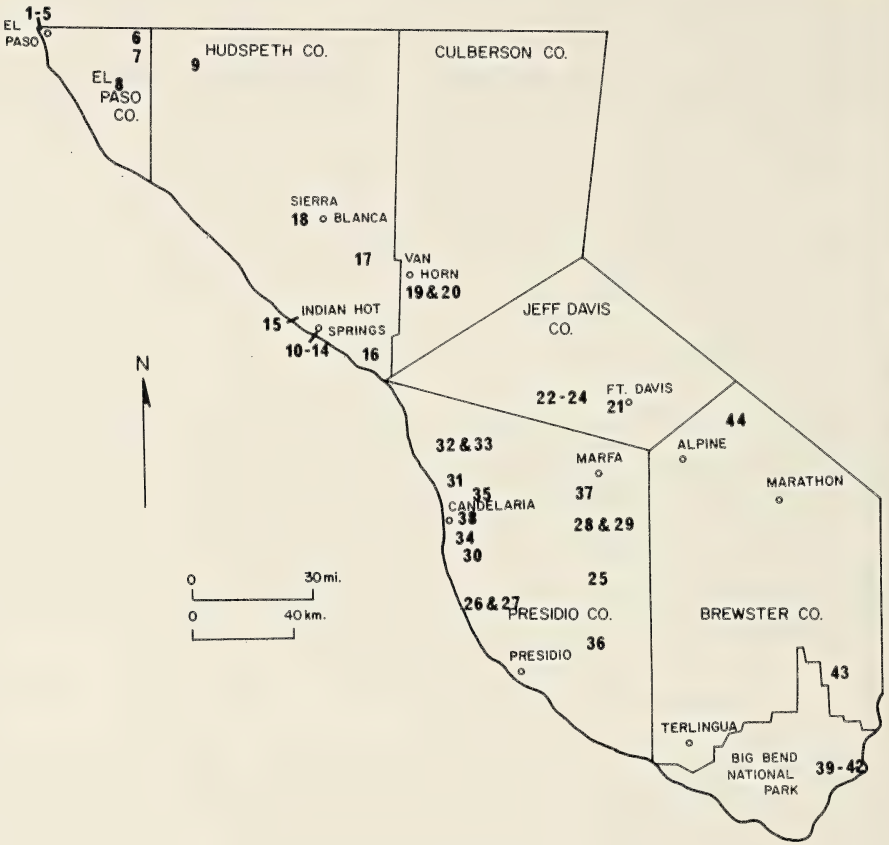

Figure 1. Thermal water occurrence, Trans-Pecos, Texas (numbers refer to thermal water locations; see text and Table 3).

The water samples were analyzed for silicon, sodium, potassium, calcium, magnesium, strontium, lithium, iron, manganese, cadmium, barium, silver, lead, zinc, and copper using atomic absorption spectrophotometry and for chloride, fluoride, bromide, and iodide using specific ion electrodes (Hoffer, 1977). This report summarizes the locations and chemistry of the thermal water occurrences.

DEFINITION OF THERMAL WATERS

The definition of thermal water is somewhat arbitrary. According to Summers (1976), subsurface temperatures are affected by climatic conditions to a depth of about 100 ft; below this depth, temperatures increase an average of 1 C (1.8 F) every 100 ft. Therefore, shallow water temperatures are influenced by surface temperatures and deep water sources by the increase in temperature toward the center of the earth.

For this report, thermal waters are defined as those that have temperatures equal to or exceeding 30 C (86 F). This value is some 6 C (11 F) above the average annual surface water temperature in west Texas and approximately 11 C (20 F) to 14 C (25 F) above the mean annual air temperature of the region. During the present study, 44 thermal water occurrences have been located.

OCCURRENCE OF THERMAL WATERS

El Paso County

Two areas in the county contain thermal waters (Fig. 1). One area occurs near El Paso just west of the Franklin Mountains where the El Paso Water Utilities Co. has encountered water averaging 33 C (91 F) in 6 wells drilled in the Mesilla bolson. Only 4 of the wells were available for sampling during this study (location 1-4, Fig. 1). All of the warm water wells produce from fine-grained sandstones of the Santa Fe Group at average depths of just over 1000 ft.

Just north of El Paso at Anthony (location 5, Fig. 1), warm water flows from a well in the alternating sandstones and mudstones of the Santa Fe Group at a temperature of 32 C (86 F).

Two warm-to-hot water wells are located in northeastern El Paso County (location 6 and 7, Fig. 1). At location 6, water at 56 C (136 F) is encountered at a depth of 595 ft, in limestone of the Hueco Group. A few miles to the south (location 7), water at 40 C (104 F) is pumped from limestone at a depth of approximately 1000 ft. At location 8, water of 33 C flows from a well drilled 400 ft into bolson-fill sediments.

Hudspeth County

Warm-to-hot waters occur at Indian Hot Springs (location 10-14, Fig. 1), Red Bull Spring (location 15), Hot Wells (location 16), a city well at Sierra Blanca (location 18), and a single well in the northwest part of the county (location 9).

A group of 8 springs occurs at Indian Hot Springs, they are located on the Rio Grande floodplain and appear to rise along the Caballo fault. The springs are located on the downthrown block a short distance from the fault trace. The waters rise along the Caballo fault and discharge at the surface in shallow dug wells (Reaser, *et al.*, 1975). Five of the 8 springs discharge warm water.

To the north, warm water from Red Bull Spring flows to the surface at the intersection of the Caballo and Red Bull faults (Jones, 1968). Hot Wells, located southeast of Sierra Blanca, are drilled into bolson-fill sediments. The 2 wells were drilled by the Southern Pacific Railroad to a depth of approximately 1000 ft. Only 1 of the wells is currently in operation and was sampled during this study.

The temperatures of thermal waters in southern Hudspeth County range from 32 C (90 F) to 46 C (115 F); the hottest waters occur at Chief Spring at Indian Hot Springs.

Culberson and Jeff Davis Counties

Two warm water wells of 32 C and 30 C are located in Culberson County a few miles southeast of Van Horn; the wells are 750 and 200 ft deep, respectively (locations 19 and 20). Three warm springs and 1 warm water well occur west of Fort Davis in Jeff Davis County; the warm springs average approximately 31 C (88 F), (locations 22-24). The springs appear to flow from faults in Tertiary volcanic rocks.

Presidio County

The largest number (14) of thermal water localities occur in Presidio County. In general, the thermal waters occur in 2 areas; one area is along the Rio Grande Valley, and the other south of Marfa (Fig. 1).

In western Presidio County, along the river valley from north to south, occur the Gulf Oil Co. wells (location 32 and 33), an unnamed spring north of Candelaria (location 31), Warm Spring (location 35), an unnamed spring east of Candelaria (location 38), an unnamed spring south of Candelaria (location 34), and 2 warm springs northwest of Presidio (locations 26 and 27).

The 2 Gulf wells were drilled to depths of 11,695 and 8,821 ft. The hot water, which reaches the surface at approximately 72 C (162 F), occurs in Cretaceous limestone at a depth of about 3000 ft (Richard Kopp, pers. comm., 1976). The limestones are capped by Tertiary volcanic tuffs. The warm springs south of the Gulf wells appear to rise along faults in the Quaternary bolson-fill.

Five thermal water occurrences (locations 25, 28, 29, 36 and 37) appear to rise along faults in Tertiary volcanic units south of Marfa.

Brewster County

Four warm springs and 2 warm water wells have been found in the county. The warm springs (locations 39-42) occur on or near the Rio Grande floodplain. The warm waters reach the surface along faults and are believed to originate at a depth of approximately 2000 ft in the Edwards Limestone (Tom Hobbs, pers. comm., 1976).

Northeast of Big Bend Park, warm water (30 C) is encountered in limestones at a depth of about 900 ft (location 43). Water at 37 C is found at 1030 ft depth in a well northeast of Alpine (location 44).

TEMPERATURES OF THERMAL OCCURRENCES

The distribution of temperatures of the thermal waters in west Texas are shown in Table 1. Temperatures range from 30 C (86 F) to 72 C (160 F); the hottest waters occur in northwestern Presidio County from the Gulf wells (locations 32 and 33). The average water temperature is approximately 37 C (99 F); median value is 34 C (93 F). The average thermal water temperature is approximately 14 C (23 F) above the average annual surface water temperature of the region.

TABLE 1

Temperatures of Thermal Water in Trans-Pecos, Texas

County	Samples	Range	Average
El Paso	8	30-58 C	37 C
Hudspeth	10	32-46 C	38 C
Culberson	2	30-32 C	31 C
Jeff Davis	4	30-32 C	31 C
Presidio	14	30-72 C	40 C
Brewster	6	30-41 C	37 C

CHEMISTRY OF THE THERMAL WATERS

The thermal waters of west Texas range from fresh to slightly salty; total dissolved solids range from 275 to 11,500 ppm. On the basis of total dissolved solids, the thermal waters of the region can be divided into 2 groups. The first group contains greater than 5000 ppm total dissolved solids; these include well waters in eastern El Paso and western Hudspeth Counties (locations 5, 6, 7, 9) and the spring waters at Indian Hot Springs in southern Hudspeth County (locations 10 to 14). The major ions in the high dissolved solids waters are sodium and chloride from possible solution of subsurface evaporite deposits. The second group contains the majority of the thermal water occurrences, those that contain less than 3000 ppm total dissolved solids.

The concentrations of 19 ions from the thermal waters and, for comparison, the average ion concentrations from all subsurface waters collected from west Texas are shown in Table 2. The following conclusions can be made concerning thermal waters of the region.

1) The average temperature of the thermal waters is 37.1 C; this value is approximately 14 C above the average temperature of subsurface waters in west Texas.

2) Sodium, potassium, silicon, chloride, lithium, fluoride, bromide, iodide, strontium, and manganese ions are more abundant in thermal waters than in nonthermal waters. These higher concentrations are probably due to the increased solubility in thermal *vs.* non-thermal waters or selective solution of subsurface evaporite deposits by thermal waters or both.

3) The concentration of calcium in thermal waters is significantly less than in nonthermal waters. This is probably due to decreasing solubility of calcium carbonate in limestones at elevated temperature.

TABLE 2

Temperature, pH, and Chemical Ions in Thermal Waters of West Texas

	Average (ppm) (37.1 C, pH 7.4)	Range (ppm) (30-72 C, pH 6-8-9.1)	West Texas Average (ppm)[1] (23.2 C, pH 7.3)
Sodium	501.80	6.00-2809.00	166.90
Potassium	31.90	0.00- 280.00	9.30
Calcium	71.60	1.00- 151.00	115.30
Silicon	23.80	9.00- 151.00	18.40
Chloride	663.00	2.00-5050.00	194.70
Fluoride	2.30	0.30- 7.20	1.40
Lithium	0.50	0.00- 2.80	0.10
Magnesium	20.10	0.00- 81.20	31.40
Bromide	1.71	0.00- 18.00	1.51
Iodide	0.05	0.00- 0.70	0.01
Iron	0.84	0.00- 16.85	1.50
Strontium	6.20	0.00- 110.00	2.90
Manganese	0.47	0.00- 18.50	0.10
Lead	0.02	0.00- 0.06	0.03
Zinc	0.14	0.00- 1.37	0.35
Copper	0.02	0.00- 0.23	0.03
Cadmium	0.00	0.00- 0.01	0.00
Silver	0.00	0.00- 0.05	0.00
Barium	0.11	0.00- 0.77	0.12
Total Dissolved Solids	2635	275-11,500	1290

[1] Average of all waters in west Texas, 1028 samples.

4) Iron, copper, zinc, and lead are less abundant in thermal waters; this is probably the result of contamination from metal pipes and casing in the more numerous sampled nonthermal wells.

5) Barium, silver, and cadmium occur in almost equal amounts in both thermal and nonthermal subsurface waters of west Texas.

Complete listings of the chemical data of each thermal water occurrence are included in Table 3.

TABLE 3

Analyses of Thermal Water Samples, Trans-Pecos Texas, in ppm

Sample No.	1	2	3	4	5	6	7	8	9	10	11
	KC-99	KC-110	KC-111	KC-112	JR-312	N-1	SN-2	JRP-324	SN-28	KC-1	KC-2
County	El Paso	El Paso	El Paso	El Paso	El Paso	El Paso	El Paso	El Paso	Hudspeth	Hudspeth (Chief Spr.)	Hudspeth (Squaw Spr.)
Location											
Longitude:	106°25′	106°37′	106°37′	106°37′	106°37′	106°06′	106°05′	106°10′	105°48′	105°13′	105°18′
Latitude:	31°55′	31°57′	31°58′	31°58′	32°00′	30°57′	31°50′	31°41′	31°45′	30°49′	30°48′
pH	7.4	9.1	9.1	8.9	7.7	7.8	7.1	7.9	6.8	6.8	7.1
Temperature	30 C	34 C	32 C	32 C	32 C	58 C	38 C	33 C	34 C	46 C	33 C
Sodium	5.50	5.90	5.70	6.10	127.20	2815.00	2580.00	280.00	914.00	2240.00	2430.00
Potassium	9.00	8.20	8.20	9.90	3.30	148.40	64.00	4.10	32.50	135.00	95.00
Calcium	2.60	0.80	1.40	3.90	22.60	274.80	242.50	17.00	131.00	135.00	138.00
Silicon	17.00	15.00	13.00	15.00	13.70	50.00	12.80	14.20	9.30	21.00	20.00
Chloride	20.50	36.00	34.00	41.00	60.00	4500.00	5050.00	296.00	1155.00	3000.00	3100.00
Fluoride	1.40	0.80	0.70	0.60	0.80	4.00	3.40	0.30	2.40	2.20	1.90
Lithium	0.10	0.10	0.10	0.10	0.10	1.30	0.90	0.10	0.40	2.70	2.80
Magnesium	10.20	0.10	0.10	0.10	0.50	57.60	81.20	0.00	71.30	38.40	37.70
Bromide	0.03	0.05	0.05	0.05	0.70	2.94	18.00	2.60	7.50	2.60	1.92
Iodide	0.01	0.01	0.01	0.01	0.03	0.20	0.02	0.07	0.06	0.04	0.06
Iron	0.06	0.01	0.01	0.01	0.03	0.03	16.85	0.01	0.67	0.27	0.19
Strontium	0.63	0.10	0.01	0.04	0.22	11.55	15.83	0.61	6.50	2.70	2.67
Manganese	0.00	0.00	0.00	0.00	0.00	0.04	0.21	0.00	0.02	0.32	0.27
Lead	0.02	0.00	0.00	0.00	0.03	0.00	0.06	0.00	0.50	0.02	0.00
Zinc	0.16	0.01	0.00	0.00	0.00	0.57	0.00	0.00	0.08	0.02	0.00
Copper	0.23	0.00	0.00	0.01	0.00	0.02	0.01	0.00	0.00	0.02	0.00
Cadmium	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Silver	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Barium	0.01	0.11	0.01	0.22	0.00	0.01	0.11	0.00	0.11	0.01	0.01
Total Diss. Solids	n.d.[1]	275	300	300	475	825	11,500	1,200	5,000	9,000	9.500
Spring or Well	W	W	W	W	W	W	W	W	W	S	S
Depth (in ft.)	1125	1090	1149	950	250	595	700	n.d.	1700	--	--

[1] Not determined, for sample locations, see Fig. 1.

Table 3 Continued.

Sample No.	12	13	14	15	16	17	18	19	20	21	22
	KC-3	KC-4	KC-5	KC-9	KC-161	JRP-231	KC-155	JRP-168	JRP-169	JRP-70	JRP-11
County	Hudspeth (Pool Spr. 1)	Hudspeth (Pool Spr. 2)	Hudspeth (Pool Spr. 3)	Hudspeth (Red Bull Spr.)	Hudspeth	Hudspeth (Hot Well)	Hudspeth	Culberson	Culberson	Jeff Davis	Jeff Davis
Location											
Longitude:	105°18′	105°18′	105°18′	105°20′	105°04′	105°01′	105°21′	104°40′	104°40′	104°30′	104°13′
Latitude:	30°49′	30°49′	30°49′	30°52′	30°44′	31°00′	31°11′	30°57′	30°57′	30°34′	30°35′
pH	7.1	7.0	7.1	8.1	8.0	7.6	7.0	7.1	7.2	7.5	7.7
Temperature	38 C	44 C	32 C	34 C	35 C	40 C	38 C	32 C	30 C	32 C	30 C
Sodium	2130.00	2210.00	2080.00	367.00	90.60	103.00	596.00	80.00	131.00	36.20	39.20
Potassium	113.00	77.00	122.00	8.80	3.50	1.80	28.00	1.80	1.00	2.40	7.90
Calcium	143.00	131.00	127.00	7.50	18.20	6.00	90.90	67.20	40.20	117.40	96.00
Silicon	25.00	22.00	25.00	19.00	9.80	10.00	13.60	14.00	26.00	28.00	32.00
Chloride	2800.00	2700.00	2775.00	88.00	14.00	2.00	608.00	62.00	38.00	12.50	18.00
Fluoride	1.70	1.60	2.10	2.60	0.30	1.70	1.10	0.90	2.70	1.60	4.00
Lithium	2.40	2.40	2.50	0.03	0.07	0.03	0.38	0.10	0.00	0.10	0.10
Magnesium	33.60	31.90	37.40	1.20	4.30	1.00	34.20	16.70	26.00	7.20	9.50
Bromide	n.d.[1]	2.29	1.85	0.14	0.24	0.21	3.20	0.10	0.42	0.03	0.03
Iodide	0.01	0.03	0.07	0.02	0.02	0.21	0.02	0.01	0.00	0.01	0.03
Iron	0.55	0.41	0.23	0.00	0.61	0.70	0.05	0.54	0.61	1.66	0.13
Strontium	2.71	2.64	2.81	0.74	0.77	0.70	2.41	0.94	0.76	0.59	0.68
Manganese	0.29	0.37	0.05	0.00	0.00	0.01	0.00	0.03	0.02	0.00	0.01
Lead	0.04	0.00	0.00	0.00	0.02	0.00	0.00	0.04	0.03	0.02	0.00
Zinc	0.01	0.00	0.01	0.00	0.07	0.03	0.04	0.59	0.15	0.05	0.00
Copper	0.00	0.03	0.00	0.00	0.01	0.01	0.00	0.01	0.03	0.08	0.00
Cadmium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Silver	0.00	0.01	0.01	0.00	0.02	0.00	0.04	0.00	0.00	0.00	0.00
Barium	0.11	0.11	0.01	0.11	0.11	0.00	0.01	0.00	0.11	0.00	0.11
Total Diss. Solids	8,400	8300	8800	1400	310	320	2700	480	570	n.d.	n.d.
Spring or Well	S	S	S	S	W	W	W	W	W	W	S
Depth (in ft.)	--	–	--	--	350	1000	1060	750	200	35	--

[1] Not determined, for sample locations, see Fig. 1.

Table 3 Continued.

Sample No.	23	24	25	26	27	28	29	30	31	32	33
	JRP-112	JRP-113	RAK-11	RAK-22	RAK-23	RAK-78	RAK-99	KC-10	KC-14	KC-16	KC-17
County	Jeff Davis	Jeff Davis	Presidio	Presidio	Presidio	Presidio	Presidio	Presidio	Presido	Presidio	Presidio
Location											
Longitude:	104°14′	104°14′	104°01′	104°28′	104°28′	104°05′	104°00′	104°34′	104°33′	104°44′	104°44′
Latitude:	30°35′	30°35′	29°51′	29°46′	29°46′	29°58′	29°56′	29°59′	30°13′	30°24′	30°24′
pH	7.8	7.5	7.3	7.4	8.0	8.3	8.4	7.6	8.9	6.9	7.4
Temperature	32 C	30 C	32 C	44 C	32 C	32 C	33 C	45 C	34 C	72 C	71 C
Sodium	40.80	42.80	117.00	17.00	7.30	19.20	117.00	167.00	104.20	143.00	146.00
Potassium	4.80	4.80	1.20	14.20	3.40	0.10	0.10	15.50	1.50	69.00	54.20
Calcium	84.00	95.00	11.40	1.70	2.30	81.80	0.60	33.00	1.00	38.00	330.00
Silicon	18.00	20.00	13.00	41.00	30.00	32.00	11.00	19.00	20.00	82.00	62.00
Chloride	18.00	15.00	9.60	103.00	139.00	9.10	16.00	64.00	15.00	289.00	237.00
Fluoride	3.20	3.00	2.40	7.20	5.80	1.00	2.60	4.20	2.60	6.30	4.40
Lithium	0.10	0.10	0.10	0.30	0.20	0.10	0.10	0.20	0.10	0.70	0.50
Magnesium	7.70	8.50	0.00	8.10	3.20	16.60	0.00	6.20	7.00	0.60	0.70
Bromide	0.05	0.05	0.04	1.60	0.10	0.03	0.04	0.07	n.d.[1]	0.25	0.40
Iodide	0.01	0.01	0.01	n.d.	0.01	0.01	0.01	0.01	n.d.	0.07	0.03
Iron	0.01	0.02	0.02	0.09	1.24	0.09	0.01	0.12	n.d.	0.12	0.10
Strontium	0.43	0.30	0.52	17.80	46.50	35.60	8.20	0.80	n.d.	0.80	0.80
Manganese	0.00	0.01	0.00	18.50	0.02	0.05	0.00	0.00	n.d.	0.01	0.00
Lead	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	n.d.	0.00	0.00
Zinc	0.02	0.05	0.00	0.01	0.01	0.12	0.01	n.d.	n.d.	0.00	0.00
Copper	0.00	0.01	0.00	0.01	0.00	0.03	0.00	0.00	0.02	0.02	0.03
Cadmium	0.00	0.01	n.d.	0.00	0.00	0.00	0.00	0.00	n.d.	0.00	0.00
Silver	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	n.d.	0.00	0.00
Barium	0.00	0.00	0.11	0.77	0.00	0.00	0.11	0.00	0.00	0.11	0.11
Total Diss. Solids	n.d.	n.d.	280.00	980.00	790.00	310.00	310.00	n.d.	n.d.	1825	1070
Spring or Well	S	S	W	S	S	W	S	S	S	W	W
Depth (in ft.)	--	–	81	–	--	200	--	–	--	8821	11,695

[1] Not determined, for sample locations, see Fig. 1.

Table 3 Continued.

Sample No.	34	35	36	37	38	39	40	41	42	43	44
	KC-20	KC-40	KC-46	KC-120	KC-147	TH-1	TH-201	TH-202	TH-203	TH-144	TH-181
County	Presidio	Presidio	Presidio	Presidio	Presidio	Brewster	Brewster	Brewster	Brewster	Brewster	Brewster
Location											
Longitude:	104°44′	104°25′	104°03′	103°49′	104°36′	102°59′	102°55′	102°57′	102°59′	102°59′	103°24′
Latitude:	30°00′	30°09′	29°43′	30°07′	30°04′	29°10′	29°11′	29°10′	29°11′	29°30′	30°30′
pH	7.4	7.5	7.4	7.2	7.1	7.7	6.8	6.9	6.9	7.2	7.0
Temperature	30 C	33 C	30 C	31 C	46 C	41 C	35 C	36 C	41 C	37 C	30 C
Sodium	110.60	66.00	120.00	98.00	159.60	98.00	68.00	93.00	94.00	70.00	623.00
Potassium	18.20	4.40	0.80	3.60	16.20	7.00	2.90	4.50	3.50	5.50	29.20
Calcium	41.00	19.00	13.00	126.40	36.90	15.70	83.00	106.20	131.00	90.20	78.60
Silicon	19.00	21.00	12.00	26.00	20.40	15.00	11.00	11.00	11.00	10.00	9.0
Chloride	58.50	9.50	25.00	92.00	61.00	60.00	64.00	68.00	82.00	75.00	990.00
Fluoride	2.90	1.00	2.30	1.90	4.00	1.80	1.70	1.70	1.70	1.60	0.50
Lithium	0.20	0.10	0.10	0.10	0.20	0.10	0.10	0.10	0.00	0.10	0.60
Magnesium	15.30	0.10	0.10	19.00	4.30	37.50	31.10	38.70	39.80	30.30	64.00
Bromide	0.11	0.04	0.04	0.11	1.00	0.11	0.05	[1] n.d.	0.10	0.11	0.90
Iodide	0.01	0.01	0.01	0.01	0.06	0.01	0.01	n.d.	0.01	0.01	0.01
Iron	2.61	1.05	0.04	0.05	0.01	0.02	0.92	n.d.	0.60	0.07	6.20
Strontium	0.90	0.26	24.60	0.39	0.59	3.65	2.69	n.d.	3.54	70.38	109.00
Manganese	0.01	0.00	0.00	0.00	0.01	0.00	0.00	n.d.	0.01	0.00	0.02
Lead	0.00	0.00	0.00	0.00	0.00	0.02	0.00	n.d.	0.02	0.04	0.00
Zinc	0.48	0.32	0.00	0.63	0.07	0.00	0.09	n.d.	0.08	0.93	1.37
Copper	0.10	0.03	0.00	0.02	0.00	0.00	0.07	n.d.	0.02	0.01	0.02
Cadmium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	n.d.	0.00	0.00	0.00
Silver	0.00	0.00	0.00	0.00	0.00	0.00	0.00	n.d.	0.00	0.00	0.01
Barium	0.00	0.11	0.00	0.00	0.00	0.00	0.00	n.d.	0.00	0.11	0.22
Total Diss. Solids	n.d.	n.d.	n.d.	790	625	n.d.	600	790	825	770	2880
Spring or Well	W	W	W	W	W	W	S	S	S	W	W
Depth (in ft.)	n.d.	n.d.	700	n.d.	138	--	--	--	--	1030	900

[1] Not determined, for sample locations, see Fig. 1.

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EFFECTS OF ALTERED CARBOHYDRATE METABOLISM ON RAT HYPOPHYSEAL GROWTH HORMONE AND PROLACTIN CONTENT

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ABSTRACT

The effects of progressive fasting, duration of insulin-induced hypoglycemia, and of short-term alloxan diabetes on hypophyseal content of growth hormone and prolactin were studied in adult male rats. Hypophyseal RNA, DNA and protein, as well as plasma glucose and protein, and liver glycogen were monitored. The results indicate that parallel GH and LTH concentrations co-existent within the rat hypophysis in response to the same carbohydrate metabolism disturbance is not an absolute finding.

INTRODUCTION

The overlapping structural and biological characteristics of growth hormone (GH) and prolactin (LTH) have received considerable investigative attention during the last 10 years. Evidence has been presented indicating that certain stimuli, e.g., β-endorphin and analogues (Dupont, *et al.,* 1977; Rivier, *et al.,* 1977), phases of the estrous cycle (Sinha, *et al.,* 1975), and insulin-induced hypoglycemia (Ajlouni, *et al.,* 1976), cause simultaneous release of GH and LTH from the mammalian anterior pituitary gland. Still other work strongly suggests that for the most part there exists a reciprocal relationship between the hypophyseal release of these 2 hormones, a relationship observed in response to TRF (Ivey and Tashjian, 1975), estradiol-17β (Macleod and Lehmeyer, 1974), and dibutyryl cyclic AMP (Macleod and Lehmeyer, 1974).

Physiologically, both GH and LTH have been found to play significant roles as regulators of mammalian carbohydrate metabolism; also, the release of both hormones may be influenced by alterations of the nutritive normalcy of the animal. Thus, the "diabetogenic nature" of endogenous GH has long been known when dogs and cats are treated chronically with GH (Houssay and Anderson, 1949; Campbell, *et al.,* 1954; Campbell and Rastogi, 1966). Also decreased peripheral glucose uptake occurs in mammals injected with GH (Evans, *et al.,* 1966),

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and very recently a subunit peptide isolated from HGH has been shown to exert both acute and long-lasting diabetic-like effects (Lostroh and Krahl, 1976) Less well documented, yet significant, are reports that LTH exacerbates the glucose intolerance of partially depancreatized dogs (Houssay, *et al.*, 1955), that hyperprolactinemia in patients is associated with typical diabetic glucose tolerance curves (Landgraf, *et al.*, 1977), and that a "diabetogenic peptide" which is structurally distinct from GH but shares impressive similarities with that of bovine LTH has been isolated and characterized (Rogol and Grissom, 1977).

From such studies as the above the possible impact of GH and LTH on mammalian carbohydrate metabolism is being brought into clearer focus. However, the impact of altered carbohydrate metabolism on hypophyseal nucleic acid and hormone profiles is unclear. Intracellular nucleic acid levels, though ancillary to hormone storage phenomena, may yield information relative to other hypophyseal cellular activity. Before examining the nature and kinetics of the duality and/or reciprocal release of GH and LTH to plasma, anterior pituitary content of nucleic acids and GH and LTH in response to well controlled carbohydrate alterations should be studied.

METHODS

Adult, male Sprague-Dawley rats were housed for at least 3 days after purchase in a room kept at 21-23 C and with a light schedule of 12 hrs on, 12 hrs off. Groups of rats were formed for study of progressive food deprivation (n = 86), insulin-induced hypoglycemia (n = 121), and alloxan-induced diabetes (n = 87). All animals were sacrificed by guillotine at 0900 ± 15 min.

Studies on fasted animals were carried out on rats weighing 200-260 g each, fasted (water *ad libitum*) for periods of 24, 48, or 72 hrs, and housed 2/metabolic cage, the latter constructed to prevent coprophagy. Periods of fasting were started such that on any given sacrifice day, all 3 fasted groups were killed. Control rats were fed Purina Lab Chow *ad libitum*.

Insulin-induced hypoglycemia was studied by injecting 1.0 U insulin, i.p./kilo body weight and sacrificing at 1/2, 1, 3, 5-1/2, and 24 hrs later. These post-insulin injection times were selected to coincide with the onset, nadir and homeostatic rebound (1/2, 1, 3 hrs, respectively) of the induced hypoglycemia, as well as with the hyperglycemic rebound (5-1/2 hrs) phase. The 24 hr group was given 4 equally spaced injections, the last of which was 6 hrs prior to sacrifice. At the time of injection food, but not water, was withdrawn from both experimental and saline-injected control rats. One control and 1 experimental rat were paired together in screen-bottom metabolic cages.

Rats weighing between 200 and 260 g each were fasted 48 hrs to reduce pancreatic and plasma insulin levels, injected with recrystallized alloxan (35 mg/kg body weight) via tail vein, and returned singly to metabolic cages where chow pellets were offered in a pulverized-porridge form. Other rats were injected with

1/4 the diabetogenic alloxan dose to establish non-diabetogenic side effects, if any, in the control group. Known amounts of feed mash were offered each day to the diabetic rats, the amount eaten calculated 24 hrs later, and the pairfed control rat offered the identical amount the next day. Daily urine volumes were collected under a layer of toluene. Diabetic rats and the pairmate controls were decapitated 1, 3 and 8 days after alloxan injection.

Blood was collected from severed neck vessels, allowed to clot at 4 C, and serum was separated the next day by centrifugation. Glucose (total reducing substances) was measured by the Somogyi-Nelson method (Somogyi, 1952), protein by the method of Lowry, *et al.*, (1951), and liver glycogen by the method of Seifter, *et al.*, (1950).

The anterior pituitary lobe was separated immediately after sacrifice, weighed on a Roller-Smith torsion balance, placed in cold large pore gel solution (1.0 mg/0.1 ml) and homogenized on a Tri-Stir apparatus. Anterior pituitary nucleic acid content was determined on pooled glands which were homogenized in 3 ml of 5% TCA. Analysis was carried out as per Schneider (1945) using DNA (Cal Biochem., salmon sperm) and RNA (Sigma, yeast) as standards and protein content by the method of Lowry, *et al.*, (1951).

Polyacrylamide gel electrophoresis of the pituitary homogenate was carried out by a modification of Nicoll, *et al.*, (1969) based on Ornstein and Davie's original method. The pH of the running (small pore) gel was 9.5, of the spacer and sample (large pore) gel 6.7, and of reservoir buffers 8.3. All gels were run at 3.0 m amps/sec for 1 to 1-1/2 hrs, stained for 1 hr, and electrophoretically destained for 10-15 min at 150 volts. Pilot studies indicated that yield of "soluble" hormone in the gel did not vary appreciably under the different experimental conditions of this study. Quantitative measurements of the hormone bands were made by densitometry on a Joyce-Loebl Chromoscan; a typical pattern is shown in Fig. 1. Areas under the hormone peak were calculated, then cut out and weighed on a semi-microbalance, the results averaged and compared with known (mg) weights of NIH GH and LTH standards on a 4 point curve. The NIH standards were a gift of Dr. Albert Parlow and were Rat GH B-1, and prolactin Rat LTH RP-1.

Statistical analysis was carried out employing the 2-tailed Students t-test for unpaired samples.

RESULTS

Fasting Rats

As can be concluded from the decrement in the body weights of 10.2% ($p<.01$), 15.2% ($p<.01$), and 18.7% ($p<.01$) for the 24, 48 and 72-hr groups, respectively, the physiological impact of progressive fasting was in proportion to the duration of the study. Absolute anterior pituitary wet weights decreased as a result of the fasting period, becoming significantly different at 72 hrs ($p<.01$). Serum glucose

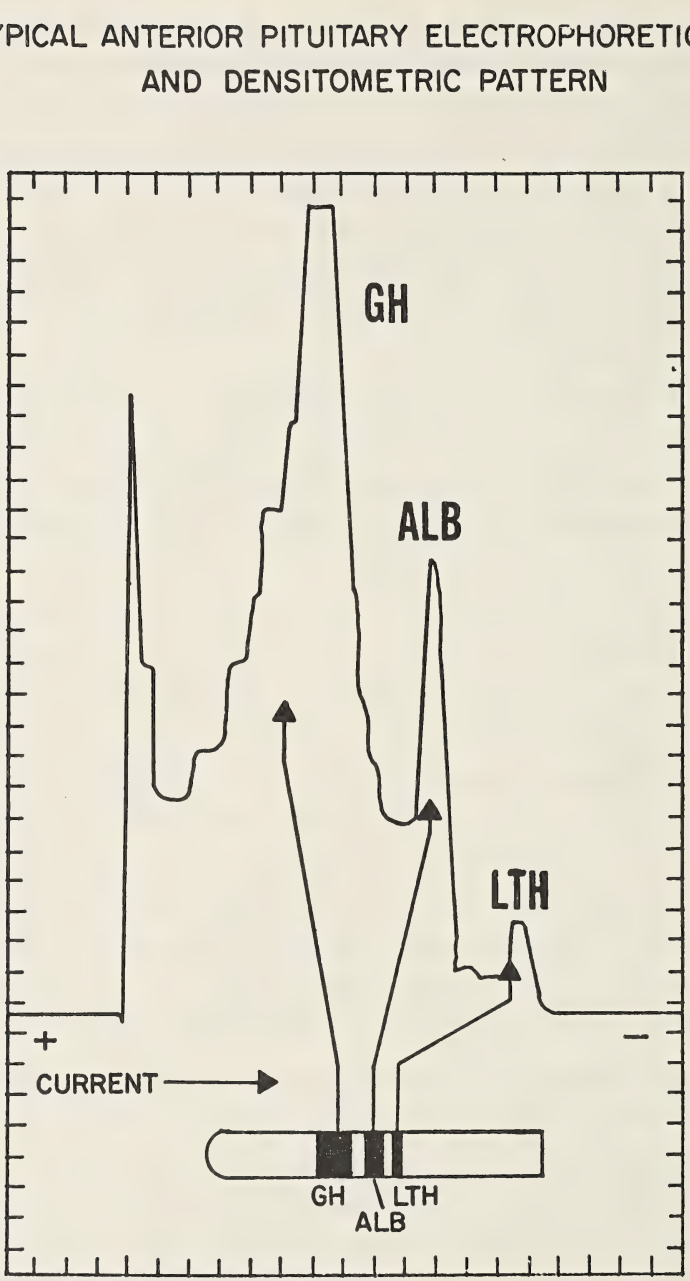

Figure 1. Typical densitometric hormone profile of GH and LTH with a schematic disc gel pattern, showing the hormone bands, positioned below. Each polyacrylamide gel represented fractionation of 1.0 mg (wet weight) of anterior pituitary tissue at pH 9.5 and with a current of 3.0 ma.

decreased markedly and significantly from fed control levels of 106 ± 3.9 mg% to 71 ± 4.6, 64 ± 6.2 and 54 ± 4.7 mg%, as did liver glycogen levels from control values of 6.6 ± 0.2% to 0.2 ± 0.1, 0.4 ± 0.1, and 0.9 ± 0.1%, respectively. All of these alterations were statistically significant at the 1% level. Serum protein increased significantly after the first fasting period from a control value of 8.1 ± 0.2% to 9.2 ± 0.1 and 9.2 ± 0.2% ($p < .05$ and $< .05$).

Table 1 presents data relative to nucleic acid and hormone content of the anterior pituitary gland of control and progressively fasted rats. It can be seen that total RNA content decreased significantly after 48 and 72 hrs of fasting.

TABLE 1

Progressive Fasting and Rat Hypophyseal (AP) Nucleic Acid, GH, and LTH Contents

Group	Ant. Pit.	Protein	RNA	DNA	RNA/DNA	Growth Hormone		Prolactin	
	mg, wet	μg/mg AP	μg/mg AP	μg/mg AP	Ratio	μg/AP	μg/mg AP	μg/AP	μg/mg AP
Control	7.4[a] ±0.3	126.0 ±12.0	7.2 ±0.4	7.1 ±0.1	1.00 ±0.04	249.0 ±7.1	36.5 ±3.1	27.0 ±3.1	3.67 ±0.40
24-Hr Fast	7.5 ±0.4	112.0 ±7.1	5.7[d] ±0.3	7.2 ±0.6	0.81[d] ±0.08	197.0[b] ±6.8	28.9[d] ±2.1	26.0 ±2.6	3.62 ±0.30
48-Hr Fast	6.8 ±0.3	124.0 ±8.8	6.2 ±0.3	8.6 ±0.8	0.73[c] ±0.06	262.0 ±7.4	37.2 ±3.2	44.0[b] ±3.2	6.51[b] ±0.90
72-Hr Fast	5.9[b] ±0.3	134.0 ±11.4	7.0 ±0.4	10.8[b] ±0.4	0.65[b] ±0.04	223.0[d] ±7.8	35.2 ±3.2	33.0 ±3.0	5.38[d] ±0.70

[a] Mean ± S.E.M. for 6-15 rats each group.
[b] p-value <.01 as compared with control group.
[c] p-value <.02 as compared with control group.
[d] p-value <.05 as compared with control group.

Total DNA content was unaltered by fasting, and only the group that fasted 72 hrs demonstrated an increase when related either to unit gland weight or protein concentration (not shown). Progressive fasting decreased the ratio of RNA/DNA significantly ($p < .05$, .02 and $< .01$). GH levels responded in a biphasic manner, decreasing at 24 hrs ($p < .01$), returning to control values at 48 hrs, only to decrease again at 72 hrs ($p < .05$). Relative to AP weight, GH levels followed a similar pattern except for the 72-hr value which was unchanged from the control level. Prolactin (LTH) levels of fasted rats were unaffected by fasting 24 hrs, but increased to supra-normal levels at 48 hrs and 72 hrs when related to AP weight ($p < .01$ and $< .05$).

Hypoglycemic Rats

Insulin-induced hypoglycemia (1.0 U/kg body weight) was without effect on body weight, serum protein, and AP tissue weight (absolute or relative). Progressive decrease in hepatic glycogen levels was apparently due to the period of fasting after insulin injection, and a significant decrease (30, 68, and 56%) in serum glucose was observed at 1/2, 1 and 24 hrs post injection ($p < .01$, $< .01$, and $< .01$) but not at 3 or 5-1/2 hrs.

The effects of hypoglycemia on pars distalis protein, nucleic acid and hormone levels are presented in Table 2. Here it is seen that insulin increased AP protein concentration ($p < .01$) in the 24-hr group, decreased total RNA 5-1/2 hrs after insulin ($p < .05$) with no change in DNA leading to a decrease in nucleic acid ratios at 5-1/2 and 24 hrs ($p < .05$ and $< .05$). Pituitary gland fresh weight was not affected by any of the treatments (5.9-6.8 ± 0.3). Growth hormone levels

TABLE 2

Insulin-Induced Hypoglycemia and Rat Hypophyseal (AP) Nucleic Acid, GH and LTH Content

Group[a]	Ant. Pit.	Protein	RNA	DNA	RNA/DNA	Growth Hormone		Prolactin	
	mg wet	μg/mg AP	μg/mg AP	μg/mg AP	Ratio	μg/AP	μg/mg AP	μg/AP	μg/mg AP
Control	6.8[b]	114.0	9.2	8.3	1.11	218.0	34.7	38.0	6.7
1-Hr	±0.4	±7.4	±0.8	±0.4	±0.10	±5.6	±2.1	±3.2	±0.4
1/2-Hr	7.0	103.0	8.1	6.5	1.20	223.0	40.3	66.0[c]	10.4[c]
Insulin	±0.4	±1.9	±0.7	±0.3	±0.10	±4.3	±2.3	±3.4	±0.5
1-Hr	6.7	127.0	9.6	8.4	1.14	256.0[c]	33.0	60.0[c]	9.3[c]
Insulin	±0.2	±2.0	±0.4	±0.2	±0.02	±4.7	±2.6	±4.1	±0.4
3-Hr	7.6	108.0	8.8	7.5	1.16	283.0[c]	39.6	45.0	6.7
Insulin	±0.3	±4.2	±0.9	±0.4	±0.07	±5.1	±2.0	±3.6	±0.3
5-1/2 Hr	6.5	132.0	7.9	8.2	0.96[e]	289.0[c]	41.1[d]	79.0[c]	25.8[c]
Insulin	±0.5	±4.6	±0.5	±0.4	±0.04	±5.2	±2.3	±4.1	±0.8
24-Hr	5.7	181.0[c]	7.6	9.9	0.77[e]	267.0[c]	48.3[c]	51.0[d]	7.8
Insulin	±0.2	±6.9	±0.2	±0.4	±0.02	±4.6	±2.6	±4.2	±0.6

[a] Additional control groups (n = 11, 13) were employed at 5-1/2 and 24 hrs post saline injection.

[b] Mean ± S.E.M. of 6-18 rats each group; each received 1 injection except the 24-hr group which received 4 equally spaced injections.

[c] p-value <.01 when compared with appropriate non-insulin control group.

[d] p-value <.02 when compared with appropriate non-insulin control group.

[e] p-value <.05 when compared with appropriate non-insulin control group.

increased after insulin injection both in absolute and in relative concentrations ($p < .01$ for 1-24-hr groups absolute; $< .02$ and $< .01$ for 5-1/2 and 24-hr group, respectively when related to AP weight). A biphasic prolactin response was observed after insulin injection, namely both absolute and relative levels of LTH increased markedly ($p < .01$ and $< .01$) returned to normal levels 3 hrs after injection, only to rise 5-1/2 and 24 hrs ($p < .01$, absolute levels only) after insulin injections commenced.

Alloxan Diabetic Rats

Onset of experimental diabetes was evaluated over the first 8 days only and was characterized by a severe curtailment ($p < .001$) in body weight gain as compared with pairfed control rats; also AP was diminished in size by 8 days ($p < .01$), serum glucose markedly increased over appropriate control values (126 *vs* 489,

99 *vs* 523, and 110 *vs* 535 mg%, respectively), liver glycogen decreased significanly in the 3-day (4.9 *vs* 2.2%) and 8-day (5.3 *vs* 2.0%) diabetic rats from non-diabetic levels ($p < .01$ and $< .01$), and urine glucose appeared in all 3 alloxan groups (0.57, 4.33 and 9.12 gm/24 hrs). As indicated by the normal values for the aforementioned parameters, the control animals appeared to be in good health from a metabolic standpoint even though they received 1/4 the alloxan dose given to the experimental rats.

The impact of early diabetes on AP protein, nucleic acid, growth hormone and prolactin levels is presented in Table 3. While a modest (relative) protein increase in AP was observed ($p < .05$) only after 8 days of diabetes, there was a significant reduction in absolute (but not relative) RNA concentrations at both 3 and 8 days ($p < .01$ and $< .01$), a decrease in absolute DNA content after 8 days,

TABLE 3

Short-Term Alloxan Diabetes and Rat Hypophyseal (AP) Nucleic Acid, GH, and LTH Content

Group	Ant. Pit.	Protein	RNA	DNA	RNA/DNA	Growth Hormone		Prolactin	
	mg wet	μg/mg AP	μg/mg AP	μg/mg AP	Ratio	μg/AP	μg/mg AP	μg/AP	μg/mg AP
Control 1-day	5.4[a] ±0.3	128.0 ±2.3	6.5 ±0.2	9.0 ±0.1	0.71 ±0.03	222.0 ±5.8	45.5 ±2.3	53.0 ±2.9	9.8 ±0.4
Alloxan 1-day	5.3 ±0.3	128.0 ±8.0	6.4 ±0.2	9.6 ±0.2	0.66 ±0.04	246.0[c] ±6.1	55.1[c] ±3.4	29.0[b] ±1.6	5.5[b] ±0.5
Control 3-day	5.8 ±0.2	141.0 ±0.9	6.3 ±0.3	10.0 ±0.4	0.64 ±0.03	223.0 ±5.8	35.1 ±3.2	25.0 ±2.0	4.3 ±0.3
Alloxan 3-day	5.6 ±0.3	136.0 ±5.1	5.2 ±0.3	10.0 ±0.5	0.52[b] ±0.06	211.0 ±6.6	38.1 ±4.1	23.0 ±1.8	4.1 ±0.4
Control 8-day	7.1 ±0.3	149.0 ±3.6	9.5 ±0.5	11.5 ±0.5	0.82 ±0.04	259.0 ±6.1	33.8 ±3.3	39.0 ±2.1	5.5 ±0.3
Alloxan 8-day	5.1[b] ±0.3	181.0[d] ±14.3	9.6 ±0.5	16.0[b] ±0.9	0.63[b] ±0.05	173.0[b] ±6.4	31.1 ±4.4	22.0[b] ±2.1	4.3[b] ±0.3

[a]Mean ± S.E.M. for 6–16 rats/group.
[b]p-values $< .01$ when compared with respective pairfed control group.
[c]p-values $< .02$ when compared with respective pairfed control group.
[d]p-values $< .05$ when compared with respective pairfed control group.

and a significant reduction in RNA/DNA ratios 3 and 8 days after alloxan injection ($p < .01$ and $< .01$). Pituitary growth hormone levels were increased (absolute and relative, $p < .02$ and $< .02$) one day after alloxan injection, returned to normal values 2 days later, only to decrease markedly ($p < .01$) on a per AP basis 8 days after injection. A high control level of prolactin was found in the 1-day group (when compared with fed controls of Table 1 and 2) but there was a progressive decrease of total LTH levels in control and alloxan groups for the remainder of the observation period. One day and eight day absolute and relative LTH levels were significantly depressed from pairfed control values ($p < .01$ and $< .01$).

DISCUSSION

The stated purpose of this investigation was to provide information as to the physiological impact of altered carbohydrate metabolism on the nucleic acid and GH and LTH content of the anterior pituitary gland in male rats. Nucleic acid data may indicate possible types of hypophyseal cellular activity induced experimentally by altered carbohydrate metabolism and may be related to whatever changes are observed simultaneously in GH and LTH levels. Alterations in these hypophyseal parameters could be generated by an effect of insulin (or relative lack of) mediated through hypothalamic influences which subsequently would be transmitted to the level of the anterior pituitary gland. Hypothalamic input to the anterior pituitary gland would most probably influence hormonogenesis therein. Alternatively, insulin presence (or absence) could affect the hypophysis directly to alter all proteinaceous components, both structural and hormonal. Accumulation of such data are a prerequisite to the future study of the duality/reciprocity of GH and LTH secretion under similar conditions, data which will compare kinetics of synthesis and release from the anterior pituitary regardless of the route of such input.

Direct effects of insulin and/or nutrient limitations on pituitary content have been reported from *in vitro* studies in which anterior pituitary glands from fasted, undernourished or diabetic rats have been incubated in the presence of labelled amino acid or glucose substrate, with and without insulin added to the incubation medium. While fasting was without effect on protein synthesis, glucose metabolism was curtailed considerably (Goodner, 1965). Our data indicate that progressive fasting caused increasingly greater reductions in the RNA/DNA ratio even though RNA concentrations were relatively stable (in face of a trend toward decreased pituitary gland size). Further direct effects on hypophyseal metabolism and synthesis have been observed by the action of insulin in stimulating lipid and protein synthesis and amino acid uptake in the rat anterior pituitary gland *in vitro*.

Results from the present *in vivo* study do not distinguish between a hypothalamic-directed effect on hypophyseal hormone content and a possible impact on the anterior pituitary gland *per se* because the hypothalamic-hypophyseal pathway was totally intact and presumably functioning. Our results are in accord with those *in vivo* studies (wherein only 1 hypophyseal secretion was under investigation) of others who have observed that protracted fasting causes reduction in GHRH (Freidmand and Reichlin, 1965; Meites and Fiel, 1965; Katz, *et al.*, 1967) and triphasic release patterns of GH in rat pituitary glands (Krulich and McCann, 1966a, Hazelwood and Barksdale, 1970; Trenkle, 1970). Simultaneous increase in LTH of the hypophysis in the present study (Table 1) probably reflects the effects of stressful stimuli which are known to decrease circulating LTH levels (Akikusa, 1971; Riegle and Meites, 1976).

Insulin excess and deprivation, both, appear to affect pituitary function directly, as well as indirectly via alteration of hypothalamic regulating factors.

Thus, while insulin increases all forms of protein synthesis in the rat pituitary gland *in vitro*, specificity of such anabolic actions must occur elsewhere, i.e., earlier in the regulator scheme (Goodner and Krouse, 1972). The strong similarity in the AP nucleic acid profile presented in Table 2 with that in Table 1 indicate that the post-prandial state of the rat was more important than was administered insulin (or attendant hypoglycemia) in effecting these alterations. However, insulin greatly increased total and relative concentrations of both GH and LTH, despite the concurrent food deprivation. Apparently, insulin may act via hypothalamic receptors (as suggested above), or at least it aids intracellular translocation of substrate for hormonogenesis. The injection of insulin *in vivo* decreases GHRH (Freidman and Reichlin, 1965; Meites and Fiel, 1965; Katz, *et al.*, 1967), increases both LTH and GH levels (Table 2; Katz, *et al.*, 1967; Hazelwood and Galaznik, 1970; Takahashi, *et al.*, 1971) and decreases plasmic LTH (Ajlouni, *et al.*, 1976).

In absence of adequate insulin the diabetic pituitary gland (*in vitro* and *in vivo*) exhibits decreased GH synthesis (e.g., Hazelwood and Hazelwood, 1964) while the addition of the pancreatic hormone increases carbohydrate metabolism, amino acid uptake, GH synthesis and LTH synthesis and release (e.g., Hazelwood and Hazelwood, 1964; Katz, *et al.*, 1967; Goodner and Krouse, 1972). Table 3 documents these effects even further. The data presented strongly suggest that the rampant gluconeogenesis attending experimental diabetes was reflected at the level of the anterior pituitary gland by decreasing RNA/gland in the 8-day diabetic group. DNA concentrations increased significantly after 8 days, effecting marked reductions in RNA/DNA ratios. Even though each control group was pairfed identical amounts of food as eaten by the respective diabetic group, the metabolic lesion was clearly evident by day 8 as far as hypophyseal proteogenesis was concerned. Also, Table 3 indicates that both LTH release and synthesis are augmented by insulin *per se* as well as by an hypoglycemia which is independent of insulin presence. Such a conclusion is consistent with recent findings indicating that insulin promotes prolactogenesis in rats (Ajlouni, *et al.*, 1976) releasing it to a greater extent than GH (Goodner and Krouse, 1972), and that an oral glucose load suppresses LTH release in normal, but not diabetic, humans (Ajlouni, *et al.*, 1976).

In conclusion, additional evidence has been offered supporting the stand that parallel GH and LTH concentrations co-existent within the rat hypophysis is not an absolute finding. Normal glucose metabolism is essential to maintaining normal hypophyseal levels of GH and LTH, and these hypophyseal contents and concentrations may react reciprocally to the same carbohydrate stress depending upon existing nutritional and temporal relationships. Kinetic studies of GH and LTH simultaneous release patterns now are required to analyze meaningfully the accumulated hypophyseal content data.

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TRANSMISSION AND SCANNING ELECTRON MICROSCOPIC STUDIES OF TENSION WOOD FIBERS USING FREEZE-ETCHING AND FREEZE-DRYING TECHNIQUES

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ABSTRACT

The gelatinous layer in *Populus tremuloides* consists of a coherent structure with cellulose microfibrils distributed parallel to one another with no apparent lamellation, and at an angle almost parallel to the long axis of the fiber cell.

INTRODUCTION

Commonly, the ultrastructure of the gelatinous layer of tension wood fibers of hardwood trees is described to consist of either a layered structure (Cote and Day, 1962; Norberg and Meir, 1966; Scurfield and Wardrop, 1962) or a coherent structure with no apparent lamellation (Mia, 1967, 1968; Morey and Cronshaw, 1968; Roberts, 1967). The former workers maintain that the gelatinous layer consists of many parallel lamellae with microfibrillar orientation almost parallel to the longitudinal axis of the fiber. While there exists no disagreement regarding the angular dispersion of the microfibrils, the latter group describe the gelatinous layer as composed of microfibrils evenly distributed throughout the entire thickness of the gelatinous layer. A third view, which has gained little support, was proposed by Sachsse (1964) to indicate that the gelatinous layer represented a honeycomb structure due to lateral anastomosing of the microfibrils.

Controversies pertaining to the ultrastructure of the gelatinous layer appear to have originated from preparative procedures followed for studying the seemingly delicate and porous gelatinous layer. It is held that the origin of the layered structure may be attributable, at least partially, to the introduction of artifacts caused by the use of methacrylate as an embedding medium (Mia, 1967, 1968). Indeed, the evidence indicates that methacrylate causes considerable alteration of the original structure of the cell walls (Mia, 1967, 1968; Heyn, 1966; Setterfield and Bayley, 1958). For this reason, the author (Mia, 1967, 1968) applied the negative staining method using the ultrathin sections of the tension wood materials embedded in epon-araldite medium, and subsequently stained in uranyl

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acetate and lead citrate. This preparation technique, more refined and sensitive than the methacrylate technique, permits direct observations of the ultrathin sections without removing the embedding medium. Our results indicate that the gelatinous layer consists of a coherent structure with even distribution of microfibrils. We believe our method of ultrathin sections with negative staining is more tenable than the one that employed replica and shadow techniques (Cote and Day, 1962; Norberg and Meir, 1966; Scurfield and Wardrop, 1962).

MATERIALS AND METHODS

In an effort to test the reliability of our findings that the gelatinous layer presents a coherent structure and not a lamellar one, the author carried out further studies on this layer using 2 ultrasensitive techniques: freeze-etching and freeze-drying, in which no chemical manipulation was needed. For freeze-etching, young live stem segments of *Populus tremuloides* were soaked overnight in 20% glycerine. From these stem segments, small pieces of tissues (about 1 sq mm), cut from the xylem adjacent to cambium, were mounted on specimen stubs, frozen in freon and in liquid nitrogen. The frozen materials were cut, etched and replicated at -160 C in a Balzar freeze-etching apparatus according to the method outlined by Branton and Moor (1964). The replicas were micrographed in the transmission electron microscope. For scanning electron microscopy, live stem segments were frozen as before but dried overnight in a freeze-drying apparatus. The specimen samples were coated with gold-palladium before examining in the Cambridge Stereo-Scanning electron microscope.

Figure 1. Scanning electron micrograph of the cross-section of the tension wood fibers showing compact gelatinous layer (G) with no lamellae; (L), cell lumen; (M) middle lamella; (S), secondary wall. X4,500.

Figure 2. Transmission electron micrograph of a replica prepared by freeze-etching technique showing longitudinal view of fiber walls containing middle lamella (M), primary wall and S_1 layer (P), S_2 layer and the gelatinous layer (G). Long arrow indicates fiber axis. X54,000.

RESULTS AND DISCUSSION

Numerous fibers containing gelatinous layers were examined in the SEM. Figure 1 represents a typical micrograph of a cross-section of stem prepared for showing the gelatinous layer. The apparently thick gelatinous layer, occurring near the lumen, can be distinguished from the outer secondary wall. The gelatinous layer, even under high magnification, reveals no lamellar structure. This layer, composed of compact microfibrils, displays a continuous phase throughout its thickness.

Figure 2 shows a transmission electron micrograph with a longitudinal view of the fiber wall prepared by the freeze-etching technique. The wall consists of a

middle lamella which lacks cellulose microfibrils. Immediately inside the middle lamella, the primary wall and the S_1 layer of the secondary wall show long strands of microfibrils with angular dispersion almost parallel to the long axis of the fiber element. The S_2 layer, usually broader than the S_1 layer, consists of microfibrils with a slight pitch and a steep angular dispersion to the longitudinal axis of the fiber. Heyn (1966) reported similar angular dispersion of the microfibrils in the secondary walls of fiber elements in a number of species of plants.

The gelatinous layer, usually broad and distinct, is deposited inner to the secondary wall (S_2 layer) as reported by a number of authors on tension wood (Cote and Day, 1962; Scurfield and Wardrop, 1962; Mia, 1967, 1968; Roberts, 1967). This layer is apparently separated from the margin of the S_2 layer by an electron dense layer (Fig. 2, short arrow). The presence of this separation zone was first reported by Mia (1967, 1968) using negative staining of the wall. The gelatinous layer revealed by freeze-etching technique displays a coherent structure with microfibrils distributed uniformly parallel to one another with no lamellation, and at an angle almost parallel to the long axis of the fiber cell.

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RESPIRATORY RESPONSES TO INSTANT THERMAL STRESS UNDER OXYGEN-ENRICHED CONDITIONS IN CENTRARCHIDS

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ABSTRACT

Opercular ventilation rates were measured by visual observation in *Lepomis cyanellus* and *Micropterus salmoides* during instant temperature stress under oxygen-enriched conditions, following collection from waters with a mean temperature of 30.8 C. Least squares linear analysis of temperature *vs*. ventilation rate for different weight categories of both species show a statistically reduced respiratory response in larger fish as compared with smaller fish. Larger fish of both species, therefore, had lower ventilation rates at higher temperatures than smaller fish, and *L. cyanellus* had lower rates than *M. salmoides* over the same thermal range. The experimental model allows rapid, repeatable estimates of stress in situations wherein complicated, automatic recording devices are not available; application of such a model in the measurement of teleost responses to toxicant and temperature coinsults seem feasible.

INTRODUCTION

Jensen (1972) has documented the successful application of instant thermal stress in fish bioassays using *Pimephales promelas*; temperature tolerance can be precisely determined with sample sizes of about 144 fish for each trial temperature. This experimental model establishes thermal lethality, necessitating use of what might be considered excessive numbers of certain rare species, long-term thermal elevations, and concomitant oxygen tension reductions resulting in oxygen insufficiency. In fact, mean survival times of *Carassius auratus* exposed to a lethal temperature in oxygen-enriched water have been cited to be 2 to 5 times longer than those for fish exposed in air-saturated water; moreover, upper lethal temperatures have been shown to be closely related to oxygen tension over a considerable range of values (Weatherley, 1970).

An experimental model was devised to employ both instant thermal stress and oxygen-enriched conditions for teleost temperature stress measurements;

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intra- and inter-species sex and weight variations were examined. Opercular rhythm, measured by visual observation (Belding, 1929; Ellis, 1937; Jones, 1947) provided a method for reducing the number of fish necessary for conducting these experiments, since a single specimen usually provided about 24 measurements. Thermal stress was measured first as temperatures decreased to the lower critical limit then as the temperatures increased to the upper critical limit. Statistical comparisons of the regression coefficients were made to assess the degree of effect induced by the first temperature change on the second. Because teleost respiratory activity and toxicity of aquatic poisons are directly related (Morgan and Kuhn, 1974; Sparks, *et al.,* 1972), such an experimental model seems suited to short-term, reliable estimates of the relative degree of stress induced by temperature and toxicant coinsults, particularly when automatic monitoring devices are not available.

MATERIALS AND METHODS

Forty-seven green sunfish (*Lepomis cyanellus*) and 67 largemouth bass (*Micropterus salmoides*) were seined from 7 collection sites on Denton and Hickory creeks south and southwest of Denton, Texas during July and August. Ambient water temperatures at the time of collection for both the surface and 1 ft beneath the surface ranged from 24.4 to 38.9 C with a mean of 30.8 C. Fish were transported to the laboratory in styrofoam coolers and maintained under constant flourescent lighting in 45.5 ℓ aquaria filled with water taken from the collection site and adjusted to the ambient temperature. Specimens were used only once and within 1 or 2 days of collection; fish were fasted before and during testing. At the initiation of testing, individual fish were weighed to the nearest 0.01 g, measured for total length in mm, and held 45 min in close confinement before being placed in a 150 ml cylindrical testing chamber fitted with a metal top through which a thermometer, aerator hose, and glass vent were inserted. For 88.1% of the specimens, this volume satisfied the prescribed chamber volume of at least 10 times the volume of the fish (Geyer and Mann, 1939). The water (150 ml) was adjusted to the ambient collection temperature (24.4 - 38.9 C) before specimen introduction and before the 10-min specimen adjustment period. Ventilation rates return to pre-stress conditions 5 min following movement of a fish to a testing chamber (Chiszar, *et al.,* 1972); therefore, this 10 min adjustment period was deemed adequate. To limit movement slightly during measurement of the number of opercular ventilations, a small piece of pliable nylon netting was placed in the testing chamber with the specimen. Both species were markedly thigmotactic and remained almost motionless, as has been reported for the brown bullhead when placed in an inverted "U" channel of appropriate size (Fry, 1957). One oxygen tablet (O-Tab, Pemble-Halverson, Inc., River Falls, Wisc. 54022) was placed in the testing chamber and minute oxygen bubbles were continuously evolved during all measurements. Oxygen is formed by the reaction of calcium

monophosphate and barium peroxide; all final products are insoluble in water except oxygen and carbon dioxide, the latter of which is 10% of gas evolved. The slight amount of hydrogen peroxide, formed is decomposed following catalyzation by magnesium dioxide. Water was saturated with oxygen (109 ± 8% saturated, mean ± standard deviation) during testing throughout the thermal range under the conditions described, as determined using a YSI (Model 54) oxygen meter.

Following the adjustment period, the testing chamber was cooled in a cold-water bath at a rate of approximately 2 C/min. Simultaneous temperature and opercular ventilation measurements were recorded at 1 min intervals until gills ceased to move for a period of 15 sec. The temperature at which this cessation occurred was assumed to be the lower critical limit (LCL or Line A). Cessation of respiration for any greater time interval usually resulted in death. After LCL was determined, the testing chamber was removed from the cold-water bath and immersed in a hot-water bath adjusted to increase the water temperature within the testing chamber at a rate of 2 C/min until opercular ventilations again ceased for 15 sec. The temperature at which this occurred was defined as the upper critical limit (UCL or Line B). In preliminary experiments, death usually occurred within 2 hrs following such treatment; therefore, all specimens were preserved in 10% formalin to prevent organ autolysis before dissection (for sex determination).

Examination of the data show that the relationship between ventilation rate and temperature during instant thermal stress is linear; least squares regression analysis was performed to establish the equation defining the line. Data were grouped according to species, sex, and size (i.e. weight) class; slopes of linear least squares line fits were categorized as line A or B according to the direction of the thermal change, as previously mentioned. The t-test was used to determine the significance of differences in slopes of the linear regression equations, thereby establishing whether such parameters as fish weight, sex, or species was influencing opercular ventilation rate during instant thermal stress under conditions of oxygen saturation.

RESULTS AND DISCUSSION

Linear least squares analysis of opercular ventilation frequency *vs.* water temperature produced correlation coefficients (r) ranging from 0.64 - 0.95 with a mean and standard deviation of 0.79 ± 0.10 and 0.83 ± 0.08 for green sunfish and largemouth bass, respectively (Fig. 1 and 2). The regression coefficients are inversely correlated with fish weight regardless of species. *L. cyanellus* females show a decrease in the regression coefficient of temperature-ventilation frequency lines from 1.61 - 0.97 ventilations/min/C; regression coefficients for males decreased from 1.84-0.93 ventilations/min/C, as weight increased from about 1-24 g. Female *M. salmoides* had regression coefficients which decreased from 2.22 to 1.33 for smallest to largest individuals; males, from 1.93-1.09 for fish 1 g or 2 g

to fish 20 g. The maximum regression coefficient observed was 2.26 for a 0.64 g juvenile bass. Therefore, not only did the larger fish of each species show decreased ventilation rates at higher temperatures but *Lepomis* had lower rates than *Micropterus*. Increased breathing rates for increasing thermal exposures has also been reported for *Cyprinus carpio* (Meuwis and Heutes, 1957), *Salmo gairdneri* (Randall and Cameron, 1973; Heath and Hughes, 1973), and *Lepomis macrochirus* (Spitzer, *et al.*, 1969).

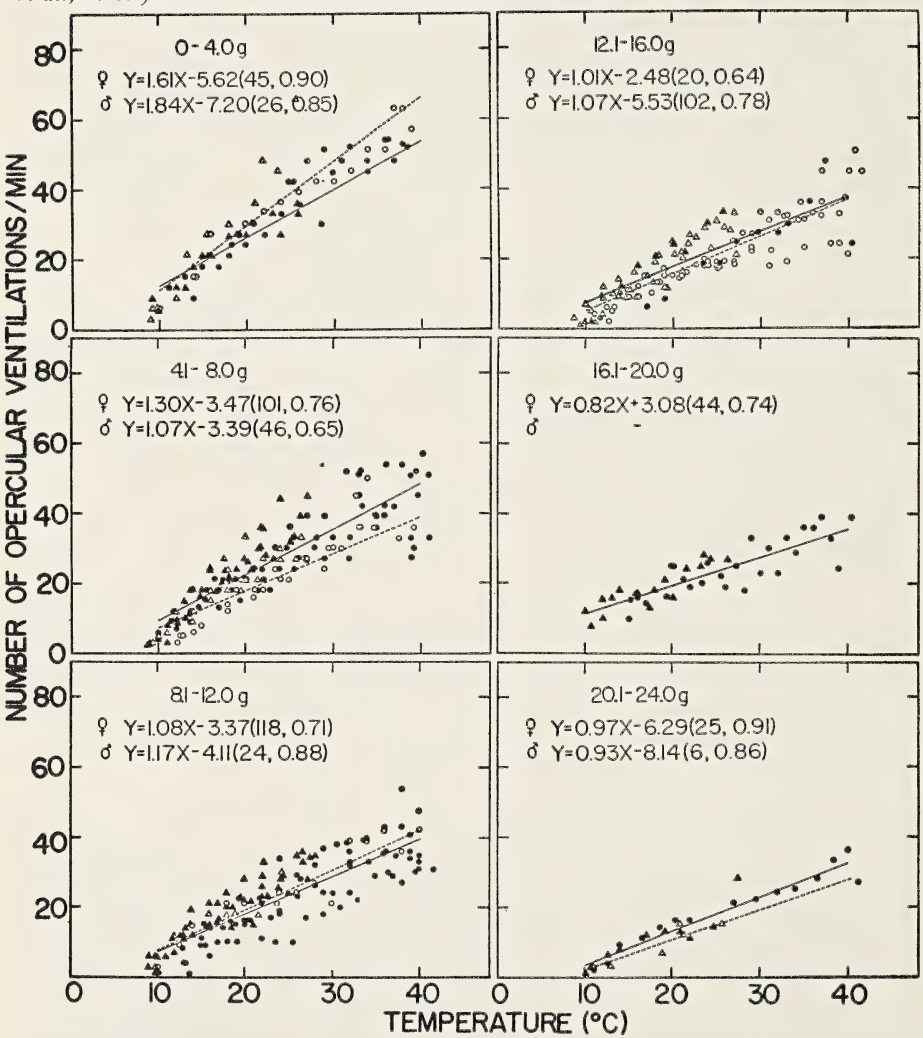

Figure 1. Thermal effects on the respiratory rate of 47 *Lepomis cyanellus* showing sexual and size class variations. Male (dotted regression lines and empty symbols) and female (solid regression lines and filled symbols) regression equations are given (beneath size class ranges) followed by number of data points and the correlation coefficient (r) for each equation (in parentheses). Triangles illustrate changes occurring from ambient temperatures to LCL (Line A) whereas, circles represent changes occurring from LCL-UCL. All data points were used in calculation of the regression line.

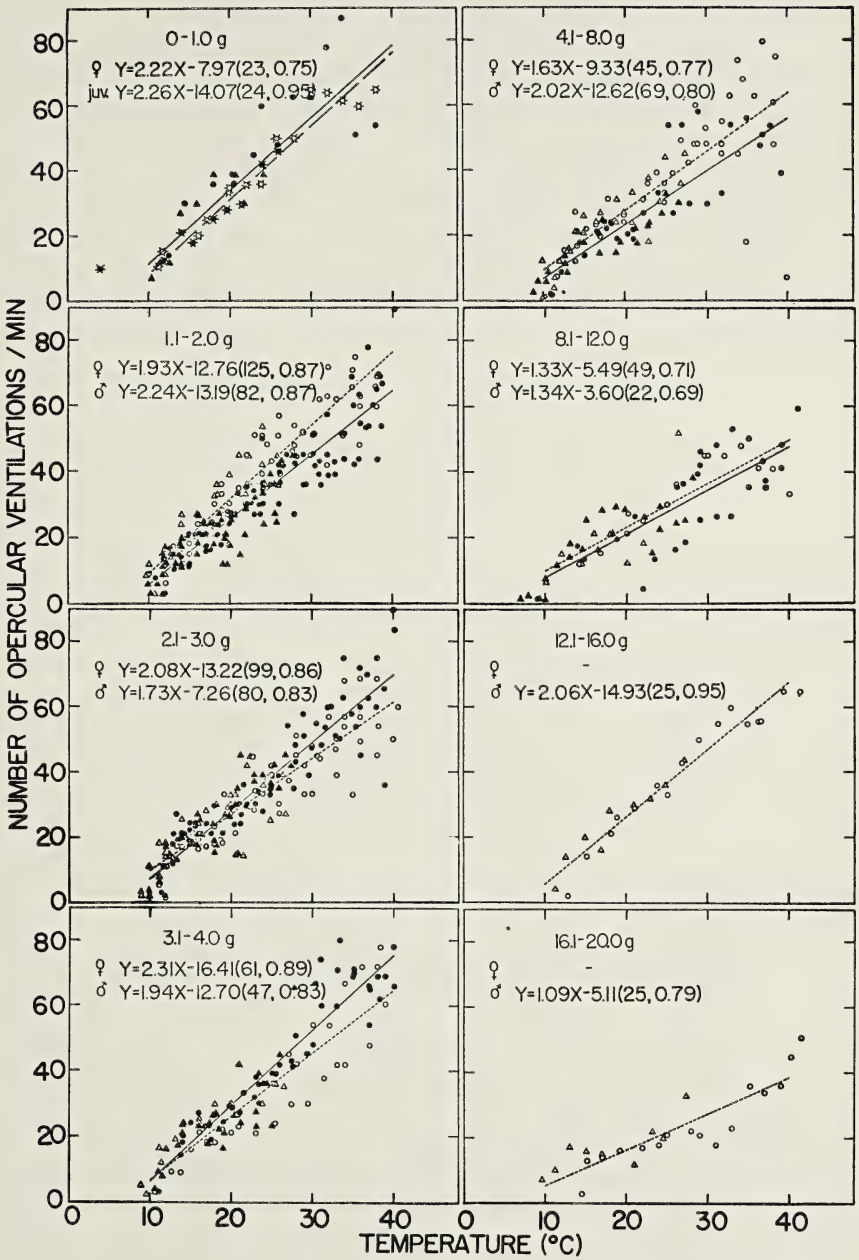

Figure 2. Thermal effect on the respiratory rate of 67 *Micropterus salmoides* showing sexual and size class variations. The term juvenile (o) was used to designate an immature fish whose sex could not be established by dissection. See Fig. 1 legend for the description of symbols.

Because statistical comparisons of temperature-ventilation frequency lines show no consistent trends between regression coefficients generated for males and females (during decreasing--Line A--and increasing--Line B--instant thermal stress), data were combined for each of the 2 species (Table 1, Column 6), All *M. salmoides* 4.1 g or larger generated regression coefficients which were significantly different from those of fish in the 1.1 - 2.0 g weight class; all 8.1 - 20.0 g *L. cyanellus* show regression coefficients significantly different from those of fish in the 4.1 - 8.0 g weight category. Therefore, bass smaller than 4.1 g or green sunfish between 8.1 g and 20.0 g could be considered for temperature-ventilation frequency slope computation without creating significant weight-induced biological variation.

TABLE 1

Comparison of Slopes from the Linear Least Squares Curve Fit of Temperature *vs.* Opercular Ventilation Rate Curves

Species	$\bar{x}b_A(s_{\bar{x},n})$[1]		$\bar{x}b_B(s_{\bar{x},n})$[2]		$\bar{x}b_{A+B}(s_{\bar{x},n})$[3]
M. salmoides wt. class (g)	♀	♂	♀	♂	♀ + ♂
0-1	2.25(-,1)	2.49(-,1)	1.94(-,1)	2.15(-,1)	2.21(.1142,4)
1-2	1.52(.1021,5)[5,6]	2.40(.1657,4)	2.02(.1340,6)[7]	2.21(.1554,4)	2.00(.1033,19)
2-3	2.09(.0946,4)	2.33(.3491,3)[6]	2.00(.1242,4)	1.71(.1071,4)	2.03(.0959,15)
3-4	2.35(.3199,2)	1.72(.0499,2)	2.30(.2113,3)	2.18(.4999,2)	2.16(.1443,9)
4-8	1.22(.0699,2)	1.82(.2430,3)	1.58(.2199,2)	2.03(.2283,3)	1.71(.1348,10)[8]
8-12	1.39(.0799,2)	2.01(-,1)	1.53(.2549,2)	1.18(-,1)	1.51(.1331,6)[9]
12-16	-	2.24(-,1)	--	2.12(-,1)	1.66(.3074,4)[8]
16-20	--	0.99(-,1)	--	1.29(-,1)	1.66(.3074,4)[8]
L. cyanellus wt. class (g)					
0-4	1.59(.2100,2)	3.35(-,1)	1.63(.0700,2)	1.76(-,1)	1.92(.2918,6)
4-8	2.07(.1616,4)[7]	1.56(0,2)	1.33(.0445,4)[4,6]	1.55(.3100,2)	1.65(.1128,12)
8-12	1.59(.0835,5)[7]	1.13(-,1)[4]	1.19(.0691,5)[7]	1.13(-,1)	1.38(.0735,12)[10]
12-16	1.86(-,1)	1.53(.2142,4)	1.28(-,1)	1.13(.1233,4)	1.38(.1293,10)[10]
16-20	1.08(.1250,2)	--	0.82(.1050,2)	--	1.03(.0888,4)[10]
20-24	1.12(-,1)	0.93(-,1)	0.93(-,1)	--	0.99(.0633,3)

[1] Mean ($\bar{x}$) slope (b) of Line A (standard error of the mean; number of specimens).
[2] Mean ($\bar{x}$) slope (b) of Line B (standard error of the mean; number of specimens).
[3] Mean ($\bar{x}$) slope (b) of both Lines A and B (standard error of the mean; number of specimens).
[4] Significantly different from opposite sex (at 5% level).
[5] Significantly different from opposite sex (at 1% level).
[6] Significantly different from second curve (at 5% level).
[7] Significantly different from second curve (at 1% level).
[8] Significantly different from $\bar{x}b_{A+B}$ of 1-2g wt. class (at 5% level).
[9] Significantly different from $\bar{x}b_{A+B}$ of 1-2g wt. class (at 1% level).
[10] Significantly different from the 4-8g wt. class (at 5% level).

Linear least squares analysis of average fish weight in the respective size classes *vs.* the regression coefficients of temperature-ventilation frequency equations shows correlation coefficients (r) of 0.61 and 0.91 for bass and green sunfish, respectively, and similar slopes of -0.038 and -0.047 (Fig. 3). Such correlations provide evidence for classification of both sympatric centrarchid species as physiologically progressive (Hart, 1952) as has been observed in some Salmonidae (Meuwis and Heuts, 1957). The lower opercular ventilation frequencies of *L. cyanellus* may indicate that they are able to acclimate to temperature changes more readily than can *M. salmoides*. Perhaps each species is adapted to slight differences in their preferences for microhabitats; physiological mechanisms may also be related to oxygen comsumption.

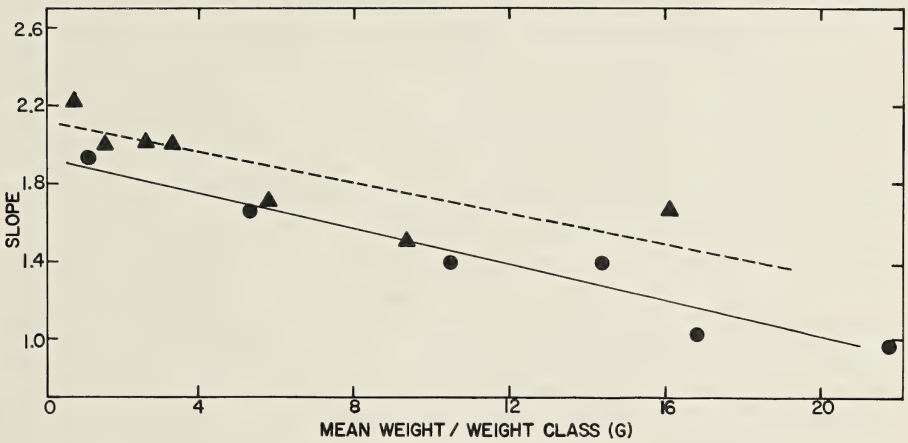

Figure 3. Linear regression lines for slope (temperature-ventilation rate) *vs.* actual mean weight of specimens within the size (i.e. weight) categories. *Micropterus salmoides* (triangles and dotted line): Y = -0.038X + 2.11 (r, 0.61) N = 67; *Lepomis cyanellus* (circles and solid line): Y = -0.046X + 1.93 (r, 0.94) N = 47. As in Table 1, 12-16 g and 16-20 g *M. salmoides* are grouped together.

Lower and upper mean critical thermal limits for green sunfish and largemouth bass following instant thermal stress were 8.3 ± 0.4 to 41.3 ± 1.2 C and 8.6 ± 0.6 to 40.4 ± 1.1 C (mean ± standard deviation), respectively. Hart (1952) found that *M. salmoides* had a thermal tolerance range of 12 - 36 C; whereas, *L. macrochirus* (closely related to *L. cyanellus*) possessed a thermal tolerance range of 11 - 34 C following the testing of 30 C acclimation groups in constant temperature baths. Upper thermal limits in the data presented here may be somewhat extended beyond normal limits because of the oxygen-enriched solutions used during testing, as has been reported to occur for goldfish (Weatherley, 1970); however, because of differences in the 2 protocols, these critical thermal limits are not directly comparable.

Greer and Gardner (1970) have shown the presence of temperature-sensitive neurons in the diencephalon of trout (*Salvelinus fontinalis*); they found that these neurons discharge at higher levels as brain temperature, rather than gill temperature, is increased and probably stimulate compensatory respiratory responses such as increased or decreased ventilation rate. Because brain temperature and corresponding increased neuronal activity lagged behind gill perfusion temperature by only 20 - 25 sec in Greer and Gardner's experiments (and assuming an association between the activity of these units in the brain and ventilation rate), it is thought that the ventilation rate changes in these sunfish and bass data accurately represent a thermal response of the 2 centrarchids that is not biased by the effect of the insulating properties of larger body size in larger fish of both species.

The experimental model used in the generation of these data seems applicable in other studies of teleost stress, such as that resulting from aquatic toxicants. Opercular rhythm varies directly with the concentration of organic [e.g. phenol, cyanide, and ammonia (Morgan, and Kühn, 1974)] or inorganic [e.g. zinc (Sparks, *et al.,* 1972) or copper and cadmium (Morgan and Kühn, 1974)] toxicants. In low-level toxicant exposure situations, (i.e. 0.1 ppm copper to *L. macrochirus*), wherein no alteration in oxygen consumption was recorded (O'Hara, 1971), opercular ventilation responses were detectable (Morgan and Kühn, 1974). The 95% confidence limits for time from introduction of toxicant to response in largemouth bass at 25 ± 1 C were less than 1 hr for 10 ppm phenol and 0.5 ppm cyanide and 2 and 7 hr for 5 ppm copper and 10 ppm ammonia, respectively (Morgan and Kühn, 1974); therefore, the experimental model used to gather these instant temperatures stress data for sunfish and bass could readily be applied in both thermal and aquatic toxicant coinsult measurements or to either stress considered alone.

This simplistic model necessitates constant surveillance during visual monitoring of ventilation frequency; however, the time and skill necessary to successfully determine opercular rhythm at 1 min intervals is minimal in comparison to that required to establish an operational automatic monitoring system.

The continuous automatic recording devices used by Sparks, *et al.,* (1972) and Morgan and Kühn (1974) were subject to baseline and amplitude variations in accordance with fish activity and distance from the electrodes, insensitivity to marked decreases in respiratory rate near death of specimens, room vibrations which necessitated special insulation (Sparks, *et al.,* 1972), and signal noise which Morgan and Kühn screened out (in part) electronically without completely solving the problem of concealment of respiratory waves during vigorous swimming of the specimen being monitored. Marginally, preliminary experiments would be most easily conducted at the more simplistic level prior to full-scale development of a sophisticated system of computerized control similar to that of Sparks, *et al.,* (1972).

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ENVIRONMENTAL FACTORS AFFECTING GROWTH AND DEVELOPMENT OF THE TEXAS MADRONE. II. INTERACTION OF LIGHT INTENSITY AND WATER STRESS

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ABSTRACT

Seedlings of the Texas madrone (*Arbutus xalapensis* H.B.K., var. *texana* Buckl.) were grown in a growth chamber for 11 weeks under the following conditions: 12-hr photoperiod, daytime temperature of 27 C and nighttime temperature of 18 C, 60% to 70% relative humidity, and light intensities of 17,200, 12,900, or 8,600 lux. Two potting media of different water-holding capacities were used.

Light intensity and water stress interacted to modify the growth and development of the seedlings. Water-stressed plants decreased in plant height, fresh weight, dry weight and chlorophyll content as light intensity increased. None of these plants developed lateral branches. Plants that were grown in the medium that had a greater water-holding capacity increased in fresh weight, dry weight, and lateral growth as light intensity increased. Chlorophyll content decreased as light intensity increased, but light intensity had no effect on plant height. All of these plants were taller and heavier than the water-stressed plants.

INTRODUCTION

Mature Texas madrone trees (*Arbutus xalapensis* H.B.K., var. *texana* Buckl.) may grow in the open under full sunlight, but madrone seedlings almost invariably grow under the protection of another tree. In the Edwards plateau region, madrone seedlings are usually found near the base of a juniper or where juniper mulch is fairly thick. Other trees that produce a heavy litter and provide light shade also will serve as "nurse trees." These observations suggest that light intensity is a

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critical factor in seedling survival, that a heavy mulch is necessary to hold sufficient water for establishment of the seedlings, or that a combination of light intensity and water relations is involved. The effects of light intensity on the growth of madrone seedlings have been reported previously (Whitenberg and Hardesty, *In Press*). In the present work the interaction between light intensity and water stress has been examined.

MATERIALS AND METHODS

Berries were collected from madrones in the Sabinal Canyon area during November, 1976. The seeds were removed and germinated in moist Fertl-Cubes (Park Seed Co., Greenwood, S.C.) or in petri dishes that contained moist peat moss. Seedlings were transferred from the petri dishes to Jiffy-7 peat pellets when the cotyledons emerged. The Fertl-Cubes and the peat pellets were then imbedded in a potting medium contained in wooden baskets (16 cm x 13 cm high). The potting medium was composed of peat moss:vermiculite:perlite: shredded juniper bark (2:1:1:1 v/v). The peat pellet held almost 3 times as much water as an equal volume potting medium (including the Fertl-Cube), and therefore provided greater moisture for the early growth of the seedling.

The baskets were placed in a growth chamber and the seedlings were grown under the following conditions: 12-hr photoperiod furnished by a mixture of cool white fluorescent tubes and incandescent bulbs; daytime temperature of 27 C and nighttime temperature of 18 C; 60% to 70% relative humidity; and light intensities of 17,200 lux (HL), 12,900 lux (ML), or 8,600 lux (LL). Each light treatment and each growth medium treatment was replicated 4 times. Plant heights were measured during the growth period, and after 11 wks the plants were harvested and were fractionated into stems and leaves. Fresh and dry weights were obtained and chlorophyll content of the fresh leaves was determined by the method of Bruinsma (1963). Meaningful root samples could not be obtained because the plant produces a very fine, fibrous root system which could not be separated from the growth medium.

RESULTS

The data for all plants are summarized in Table 1. Plants started in peat pellets (non-stressed) and plants started in Fertl-Cubes (water-stressed) were significantly different in the height, fresh weight, dry weight, and chlorophyll content at all light intensities. Plants that had adequate water averaged about twice the height of the water-stressed plants, and had about 6 to 20 times the fresh weight and 6 to 30 times the dry weight of the water-stressed plants. The non-stressed plants produced a significantly greater percentage of dry matter than did any of the other plants. Both water-stressed and non-stressed plants decreased in chlorophyll content as light intensity increased, but the response was greater in the non-stressed plants.

TABLE 1

Effects of Light Intensity and Water Stress on the Growth and Chlorophyll Content Content of Texas Madrone Seedlings.[1]

Light Treatment	Plant Heights cm	Fresh Wt. mg	Dry Wt. mg	Avg. Dry Wt. %	Chlorophyll mg/g tissue
		Water-stressed Plants			
17,200 lux	3.1[2] ± 0.2	145 ± 5	43.5[2] ± 1.0	30	1.30[2] ± 0.06
12,900 lux	3.7[2] ± 0.3	153 ± 7	47.4[2] ± 1.3	31	1.48[2] ± 0.04
8,600 lux	5.0[2] ± 0.4	275[2] ± 18	82.5[2] ± 5.3	30	1.76[2] ± 0.09
		Non-stressed Plants			
17,200 lux	8.6 ± 0.5	3,123[2] ± 75	1,289[2] ± 32	41	0.86[2] ± 0.03
12,200 lux	8.0 ± 0.3	2,468[2] ± 61	969[2] ± 26	39	1.16[2] ± 0.08
8,600 lux	9.2 ± 0.6	1,757[2] ± 57	528[2] ± 19	30[2]	2.17[2] ± 0.09

[1] Each mean (± standard error) is the average of 4 replications.
[2] Means that are significantly different ($P < 0.05$).

The water-stressed plants decreased in height, fresh weight, dry weight and chlorophyll content as light intensity increased. Light intensity did not affect the ratio of dry tissue to fresh tissue. The chlorophyll content of the non-stressed plants also decreased as light intensity increased, but plant height was not affected, and fresh weight, dry weight, and the ratio of dry weight to fresh weight increased as light intensity increased.

Photomorphogenetic differences among the plants were also observed. Non-stressed plants of the HL and ML treatments developed lateral branches, but none of the other plants produced branches. Plants of the HL treatment developed an average of 7 branches (0.3 to 2.6 cm long), and plants of the ML treatment averaged 3 branches (0.4 to 1.7 cm long). All plants of the HL and ML treatments had slimmer, thicker, and more leathery leaves than did the plants of the LL treatment.

DISCUSSION

The data indicate that light intensity is an important factor in the growth and development of Texas madrone seedlings. However, there is an interaction between light intensity and water availability, and light intensity appears to be a critical factor only when the seedlings are water stressed. Higher light intensities introduce a second stress, photobleaching, which in conjunction with water stress severely limits growth and contributes to seedling death. The normal range of the Texas madrone is in arid or semi-arid regions of Texas, and seedlings are normally water stressed. A "nurse tree" provides partial shade and thereby reduces the light stress, which in turn allows the seedlings to survive when water is

limiting. Such a hypothesis is compatible with our observation (Whitenberg and Hardesty, 1978) that madrone seedlings that grow under natural conditions are found where the light intensity is 6,500 to 10,000 lux. Since water-stressed plants and non-stressed plants were photobleached by the HL and ML treatments, photobleaching apparently is less important if there is adequate water.

The morphogenetic differences in the leaves of plants that received different light treatments are typical of plants adapted to sun or shade (Salisbury and Ross, 1969). Leaves of plants grown under low light intensity commonly are wider, thinner, and have more chlorophyll/unit weight than do the leaves of similar plants grown under higher light intensities. Photosynthetic efficiency is also less in plants adapted to shade. The data do not suggest any explanation for the development of lateral branches on the non-stressed plants of the HL and ML treatments. Possibly, higher light intensities reduce apical dominance or provide the photosynthates necessary for lateral growth.

ACKNOWLEDGEMENTS

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QUALITY OF WATER IN SELECTED WELLS OF THE LINN–FAYSVILLE GROUND WATER RESERVOIR, SOUTH TEXAS

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ABSTRACT

The Linn-Faysville Ground Water Reservoir is an important source of irrigation and domestic water in the Linn-Faysville district of Hidalgo County. The ground water of the reservoir contains greater than 250 ppm of sodium (Na^+) and chloride (Cl^-), making the water hazardous for agricultural purposes. The levels of nitrate (NO_3^-) and fluoride (F^-) make the water undesirable as drinking water; however, there is no suitable drinking water substitute for most rural families in the area. The ground water, although highly mineralized is essentially unpolluted based on the absence of toxic metals and nitrate concentrations.

INTRODUCTION

The Linn-Faysville area of Hidalgo County, in the lower Rio Grande valley, south Texas (Fig. 1), is heavily agricultural and noted for its high production of cotton, wheat, and grain sorghum. Most of the water used to irrigate these crops is ground water, withdrawn from a shallow, generally unconfined aquifer which underlies the area. The shallow aquifer is the primary source of domestic drinking water for many rural families and is also used to a limited extent as a municipal water supply. The chemical quality of the area's ground water has become of increased interest since the area was included into the Lower Rio Grande Valley 208 Planning Area, as declared by the Environmental Protection Agency under Public Law 92-500, section 208, June 6, 1975. Unfortunately the chemical make-up of the area's ground water has not been evaluated for approximately 20 years. The purpose of this paper is to update the existing ground water quality data base of the Linn-Faysville area and comment on the advisability of using the area's ground water for agricultural purposes and as domestic drinking water. The limited number of wells examined preclude any firm conclusions from being made; however, the data are useful as an indicator of quality of the area's ground water at this point in time.

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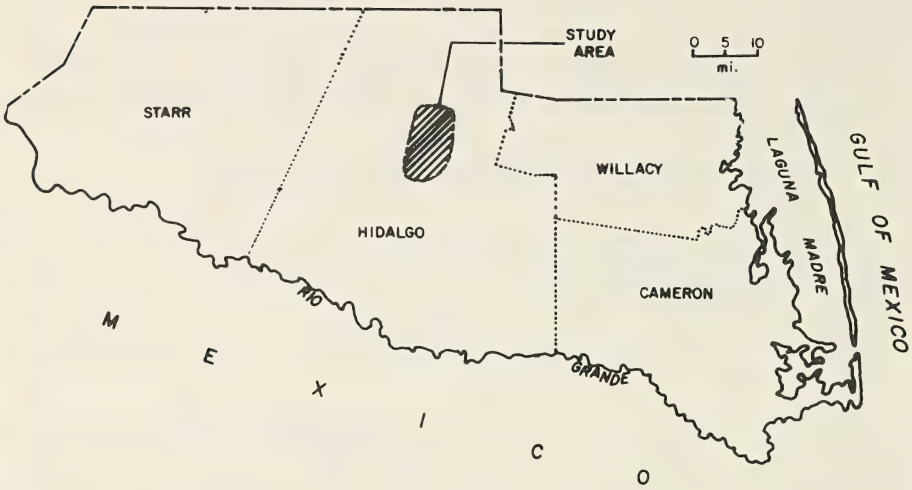

Figure 1. Lower Rio Grande valley region.

GENERAL HYDROGEOLOGY

On the basis of geology and water quality Baker and Dale (1961) hydrologically differentiated the area from surrounding areas and applied the term "Linn-Faysville Ground Water Reservoir." The lithology of the reservoir exhibits considerable lateral variation but, in general, consists of a series of red, pink, orange and gray clays, with chert and limestone pebble conglomerates interbedded with sands and sandstone (Trowbridge, 1932; Weeks, 1945). The reservoir is unconfined over most of the area; however, localized perched water table or leaky artesian conditions may exist depending on the presence or absence of the numerous clay beds and thin lenses of caliche.

METHODS AND MATERIALS

A total of 18 samples from 9 locations were collected from the study area (Fig. 2). This corresponds to 1 well/5 mi^2. However, in the northern parts of the reservoir, where agricultural and domestic use of the reservoir is greatest, the sampling interval is approximately 2 mi^2. In collecting the samples the pump was turned on for a sufficient period of time to clear the casing and insure that a fresh sample was taken. Immediately after collection 1 of the 2 samples was acidified with nitric acid to prevent the metals from plating onto the container walls; the second sample was left untreated.

Figure 2. Detail of study area with sampling sites.

Sodium adsorption ration (SAR) was determined by calculation from the analyses. All major cations as well as the trace metals were determined by atomic absorption spectrophotometry (AAS). Trace metals were concentrated by the MIBK-APDC organic extraction method (Brooks, *et al.*, 1967) and mercury was determined by flameless AAS (Hatch and Ott, 1968). Chloride and bicarbonate (HCO_3^-) were determined by titration (American Public Health Assoc., 1976). The remaining parameters were determined using a Hach Model DR-EL/2 visual spectrophotometer.

RESULTS

The results of the analyses are given in Table 1. Ions found at detectable concentrations at all sample locations include bicarbonate, calcium (Ca^{2+}), chloride, fluoride, magnesium (Mg^{2+}), potassium (K^{+}), silica (SiO_2), sodium, nitrate, strontium (Sr^{2+}), and sulfate (SO_4^{2-}). Nitrite (NO_2^{-}) was found at only 2 locations. Zinc (Zn^{2+}) was detected at all sampling sites and copper at 8 of 9 locations. No other trace metals were detected. All undetected metals, including cadmium, chromium, cobalt, lead, manganese, nickel, and silver are considered to be less than 1.0 μg/L (PPb). Mercury is considered to be below 0.1 μg/l.

TABLE 1

Chemical Analysis, Specific Conductance, Temperature and Sodium Adsorption Ratio for Selected Wells in the Linn-Faysville Ground Water Reservoir[1]

RRN[2]	Ca^{+2}	Mg^{+2}	Na^{+}	K^{+}	Sr^{+2}	Cl^{-}	SO_4^{-2}	F^{-}	HCO_3^{-}	NO_3^{-}	NO_2^{-}	Zn^{+2}	Cu^{+2}	SiO_2	SC (μΩ/cm)	Temp (C)	SAR
1	29	20	508	9.0	0.65	525	180	1.65	438	2.0	0.00	0.18	0.05	10	2833	27	17.7
2	59	28	325	7.5	0.45	380	150	1.50	353	1.8	0.00	0.04	0.02	21	2267	27	8.7
3	27	28	230	7.5	0.50	205	120	1.55	341	7.0	0.00	0.04	0.01	15	1616	26	7.4
4	55	45	250	9.0	1.15	300	175	1.10	341	8.0	0.00	0.13	0.02	14	2167	27	6.1
5	54	37	305	10.0	0.65	380	145	3.00	366	2.0	0.00	0.08	0.06	16	2300	27	7.8
6	22	67	1205	67.0	1.30	1300	500	1.30	877	5.7	0.02	0.03	0.01	11	7467	27	28.7
7	60	40	290	9.0	1.30	425	80	1.70	366	2.1	0.00	0.18	0.01	16	2400	27	7.1
8	37	41	408	13.0	0.60	420	160	1.50	414	4.5	0.03	0.03	0.01	12	2467	27	11.0
9	15	69	1085	19.0	1.00	1340	265	2.40	695	4.3	0.00	0.01	–	10	6450	28	26.3

[1] Units are mg/l unless otherwise indicated.

[2] Report reference number.

DISCUSSION

The calculated values of SAR, a commonly used measure of the sodium hazard of irrigation water, ranged from 28 (very high) to 5 (low). The high sodium concentrations in conjunction with the low calcium and magnesium concentrations present a serious hazard to many types of crops. In addition, the high chloride concentrations of the ground water would certainly increase the salinity of the soil irrigated with water from the Linn-Faysville reservoir if corrective measures are not taken.

To prevent serious cutbacks in production that result from excessive salinity in the root zone, surplus irrigation water is often used to "flush" the contaminants

from the root zone into the ground water system. This practice has been reported to result in a steady increase in the concentration of contaminants in the ground water (Helweg, 1977). As irrigation water is used in this process, some of the water evaporates or is transpired by the plants. The transpired and evaporated water is very low in dissolved solids while the concentration of solids in the remaining water has increased. As a result of this process the dissolved solid content of the ground water is slowly increased from year to year with each application of irrigation water. Eventually the point may be reached when the Linn-Faysville Ground Water Reservoir will be severely limited as a source of water for most agricultural purposes.

As a domestic water supply for individual families, the major chemical constituents limiting the use of the reservoir are nitrate, fluoride and nitrite. The toxic potentials and physiological effects of these contaminants have been well documented (EPA, 1976). Excessive nitrate and nitrite produce methemoglobinemia ("blue-baby") in infants who ingest excessive amounts. The EPA limit proposed for these 2 anions is 10 ppm and 0.1 ppm respectively. At the levels present in the reservoir (1.3-3.0 ppm) fluoride is not toxic; it may, however, produce a mottling of the teeth. Other ions present in the reservoir such as chloride, zinc and copper (Cu^{2+}), are not in concentrations high enough to be toxic, but may impart undesirable characteristics to the water such as color, taste or odor.

Although highly mineralized, the ground water of the area does not appear to be affected by man-made contamination at this time. The relatively low nitrate concentration (less than 10 ppm) indicates a lack of excessive pollution from septic tanks, landfills and fertilizer, each of which are reputed to be capable of producing significant nitrate pollution of ground water (Saffigna and Keeney, 1977). Heavy metals are also indicative of man-made contamination and the absence of such metals from the samples supports the contention that the ground water is essentially free of man-made pollutants.

SUMMARY

Ground water of the Linn-Faysville Ground Water Reservoir is highly mineralized and exhibits considerable lateral variation. The degree of mineralization limits the usefulness of these waters for irrigation. In addition, the levels of nitrate and fluoride may make the consumption of the water harmful. Except for zinc and copper, which are not toxic at the levels present, the reservoir is free of heavy metal contamination. However, it is not recommended that dependence on the Linn-Faysville Ground Water Reservoir be decreased. Instead it is hoped that the ground water of the reservoir will continue to be monitored on a regular basis so that the reservoir may be properly managed and more fully utilized.

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SYNTHESIS AND MICROBIOLOGICAL PROPERTIES OF 2-IODOTYROSINE AND RELATED COMPOUNDS

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ABSTRACT

2-Iodotyrosine, the remaining analog in a series of 2-monohalogenated tyrosines has been synthesized and tested for its microbiological activities. 2-Iodo-4-methoxybenzyl chloride was condensed with ethyl acetamidomalonic ester in ethanolic sodium ethoxide to afford ethyl α-acetamido-α-(2-iodo-4-methoxybenzyl) malonate, which was hydrolyzed with hydrochloric acid to yield 2-iodo-4-methoxyphenylalanine. Demethylation of the latter compound by treatment with boron tribromide or hydrobromic acid gave 2-iodotyrosine. 2-Iodotyrosine inhibited the growth of *Lactobacillus plantarum* 8014 and *Streptococcus faecalis* 8043 at concentrations of 20 and 200 μg/ml, respectively, and the growth inhibition of *L. plantarum* was reversed competitively by tyrosine over a 100-fold range of increasing concentrations with an inhibition index of about 1000. However, 2-iodotyrosine was not inhibitory to the growth of *Escherichia coli* 9723 at concentrations of 200 μg/ml. Under the same assay conditions, neither 3-iodotyrosine nor 3,5-diiodotyrosine were active as growth inhibitors of these microorganisms.

INTRODUCTION

Among a group of 2- and 3-halogenated tyrosines studied earlier, the 2- and 3-fluorotyrosines were the most effective analogs to specifically inhibit tyrosine utilization in a competitive manner for *Escherichia coli* 9723, *Lactobacillus plantarum* 8014, and *Streptococcus faecalis* 8043 (McCord, *et al.*, 1975). Although 2-fluorotyrosine was similar to 3-fluorotyrosine in its inhibitory effects upon the growth of these microorganisms, the 2-chloro- and 2-bromotyrosines were far more effective inhibitory analogs of tyrosine than the corresponding isomeric 3-chloro- and 3-bromotyrosines (McCord, *et al.*, 1975).

Because of the general inhibitory activities of the 3 2-halotyrosines, it was of interest to complete the series by preparing the remaining unreported analog, 2-iodotyrosine, for a comparative study of its microbiological activities with those of some structurally related compounds. Accordingly, in this study, 2-iodotyrosine and 2-iodo-4-methoxyphenylalanine were synthesized and assayed for their microbiological activities in *E. coli, L. plantarum,* and *S. faecalis*

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in comparison with those of 3-iodotyrosine and 3,5-diiodotyrosine. Although not inhibitory toward *E. coli*, 2-iodotyrosine inhibited the growth of *S. faecalis* and *L. plantarum*, and its growth inhibition of the latter microorganism was competitively reversed by tyrosine. In contrast to these microbiological results, the 3-iodo- and 3,5-diiodotyrosines showed no inhibitory activity in the same assay organisms.

The sequence of reactions used in the synthesis of 2-iodotyrosine (*4*) is shown in Fig. 1. 2-Iodo-4-methoxybenzyl chloride (*1*) was synthesized by direct chloromethylation of *m*-iodoanisole with chloromethyl methyl ether. The chloride *1* was then condensed with ethyl acetamidomalonate (EtAAM) in the presence of sodium ethoxide to yield ethyl α-acetamido-α-(2-iodo-4-methoxybenzyl) malonate (*2*). In Method A, hydrolysis and decarboxylation of the malonic ester *2* was effected in refluxing hydrochloric acid to give 2-iodo-4-methoxyphenylalanine (*3*). By following a previously reported procedure (McOrmie, *et al.*, 1968), the latter compound *3* was demethylated by treatment with boron tribromide to form the dibromoborate intermediate which was hydrolyzed and then neutralized to yield the desired 2-iodotyrosine (*4*).

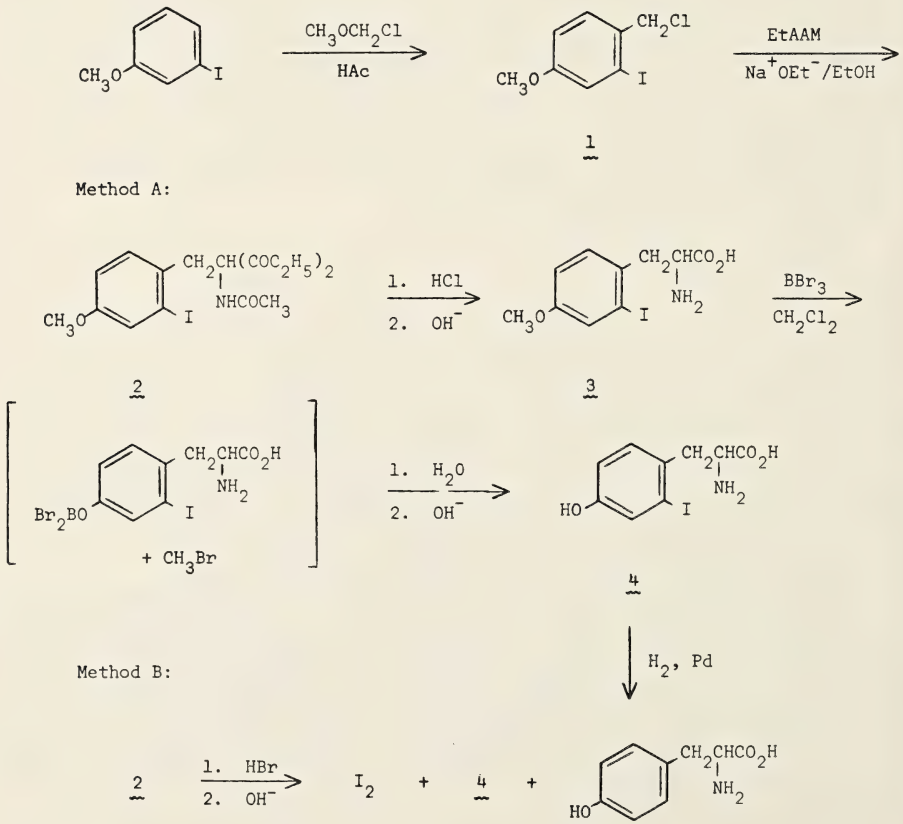

Figure 1. The sequence of reactions used in the synthesis of 2-iodotyrosine *(4)*.

In Method B, an initial attempt was made to obtain the 2-iodo analog *4* directly from the malonic ester *2* by refluxing in concentrated hydrobromic acid in the same manner which was successful in synthesizing the other 2-halotyrosines (McCord, *et al.*, 1975). Under these conditions, however, extensive deiodination occurred concomitantly with demethylation to afford tyrosine and low yields of 2-iodotyrosine (*4*).

2-Iodotyrosine obtained from either Method A or B was isolated in the form of its free base monohydrate as confirmed by elemental analysis. The structure of the amino acid *4* was established by its conversion to tyrosine by catalytic hydrogenation to further verify the orientation of the chloromethylation of *m*-iodoanisole in the initial step. The 2-iodo analog was characterized by its melting point, chromatographic mobilities, and IR and NMR spectra.

The effects of 2-iodotyrosine, 2-iodo-4-methoxyphenylalanine, 3-iodotyrosine, and 3,5-diiodotyrosine upon the growth of *E. coli* 9723, *L. plantarum* 8014, and *S. faecalis* 8043 were compared to those of the previously reported 2-chlorotyrosine (McCord, *et al.*, 1975) as summarized in Table 1. The latter compound was selected as the reference standard for the purpose of comparing the microbiological results of this study with those obtained from previous work on the other 2-halotyrosines (McCord, *et al.*, 1975) and some β-(1-halo-2-naphthyl) alanines (McCord, *et al.*, 1976).

TABLE 1
Relative Inhibitory Activities of 2-Iodotyrosine and Related Compounds in Various Microorganisms

	Minimum Inhibitory Concentration (μg/ml)[1]		
Compounds	*E. coli*[1] 9723	*L. plantarum*[2] 8014	*S. faecalis*[3] 8043
2-Iodotyrosine (*4*)	>200.00	20.00	200.00
2-Iodo-4-methoxyphenylalanine (*3*)	>200.00	>200.00	>200.00
3-Iodotyrosine	>200.00	>200.00	>200.00
3,5-Diiodotyrosine	>200.00	>200.00	>200.00
2-Chlorotyrosine	2.00	0.02	6.00

[1]Minimum inhibitory concentration required for complete inhibition of growth.
[2]Growth media and assay conditions as described in Experimental Section except that 5 μg of phenylalanine was added to each assay tube.
[3]Growth media and assay conditions as described in Experimental Section.

None of the 4 iodinated amino acids were active up to a concentration of 200 μg/ml in *E. coli*. Under the specific assay conditions described in the experimental section, the 2-iodo analog was the only one of the compounds which

was inhibitory to the growth of both *L. plantarum* and *S. faecalis*. 2-Iodo-4-methoxyphenylalanine at moderately high concentrations inhibited the growth of only *L. plantarum*, whereas neither 3-iodotyrosine nor 3,5-diiodotyrosine showed any inhibitory activity in the assay organisms.

The reversal of 2-iodotyrosine inhibition by DL-tyrosine was studied more extensively in *L. plantarum* as indicated in Table 2. Growth inhibition by 2-iodotyrosine was reversed in a competitive manner over a 100-fold range of increasing concentrations of tyrosine with an inhibition index (ratio of the concentration of 2-iodotyrosine to the concentration of tyrosine required for complete inhibition of growth) of approximately 1000. Thus 2-iodotyrosine, though less effective than 2-chlorotyrosine, was a specific and competitive antagonist of tyrosine in *L. plantarum*.

This

TABLE 2

Reversal of 2-Iodotyrosine Inhibition by DL-Tyrosine in *Lactobacillus plantarum* 8014[1]

2-Iodotyrosine μg/ml	Absorbance Readings,[2] DL-tyrosine, μg/ml					
	0.00	0.02	0.06	0.20	0.60	2.00
0	.55	.54	.54	.54	.52	.54
2	.25					
6	.07	16	38	50		
20	.00	05	.09	.21	.54	.50
60		.00	.00	.05	.39	.52
200			.00	.00	.03	.54
600					.00	.08

[1] Growth media and assay conditions are described in the Experimental Section.

[2] A measure of culture turbidity in which absorbance readings of 0.50, 0.40, 0.30, 0.20, and 0.10 are equivalent to 2.19, 1.65, 1.13, 0.56, and 0.075 mg of dry wt of cells/ml of culture, respectively.

This study further shows that the size and the position of the halogen in the benzene ring of tyrosine have a pronounced effect on the ability of such analogs to inhibit tyrosine utilization, and it also makes available the last of the series of 2-halogenated tyrosines for biological and biochemical studies. It also appears that this is the first report which demonstrates *in vitro* antibacterial activity of an iodo analog of an aromatic amino acid.

EXPERIMENTAL SECTION

General

Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. The R_f data were determined using the ascending technique in the solvents indicated, and ninhydrin reagent was used for development of the spots.

Infrared spectra were recorded on a Beckman Model IR-10 spectrophotometer (KBr) and were calibrated with polystyrene film. The NMR spectrum of 2-iodotyrosine (30 mg) was recorded in about 0.5 ml of 20% DCl in D_2O solution with 1% sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the reference standard. Elemental analyses were performed by the M-H-W Laboratories, Garden City, Michigan.

2-Iodo-4-methoxybenzyl Chloride (1).

To a solution of 10.3 g (0.13 mol) of chloromethyl methyl ether in 175 ml of glacial acetic acid was added 15.0 g (0.06 mol) of *m*-iodoanisole. After stirring at 55 C for 48 hr, the mixture was poured over ice to give a waxy solid. Filtration yielded 13.4 g (72.3%) of product. Recrystallization from chloroform gave an analytical sample, mp 50-52 C. Anal. Calcd. for C_8H_8ClIO: C, 34.00; H, 2.86. Found: C, 33.85; H, 2.90.

Ethyl α-Acetamido-α-(2-iodo-4-methoxybenzyl) malonate (2).

To a solution of 4.24 g (0.02 mol) of ethyl acetamidomalonate in 50 ml of absolute ethanol containing 0.45 g (0.02 mol) of sodium was added 5.0 g (0.018 mol) of 2-iodo-4-methoxybenzyl chloride. The reaction mixture was allowed to stir at 25 C for 5 hr followed by reflux for 2 hr. The sodium chloride was removed by filtration, and the resulting filtrate was reduced in volume *in vacuo* to 1/2 of the original volume. An equal portion of water was added to effect precipitation. After chilling overnight, the precipitate was filtered yielding 6.47 g (78.9%) of product. An analytical sample, mp 131-133 C, was obtained by recrystallization from methanol-ether. Anal. Calcd. for $C_{17}H_{22}INO_6$: C, 44.07; H, 4.80; N, 3.02. Found: C, 44.34; H, 4.63; N, 3.24.

2-Iodo-4-methoxyphenylalanine (3).

A 6.0 g sample (0.013 mol) of ethyl α-acetamido-α-(2-iodo-4-methoxybenzyl) malonate was hydrolyzed in the presence of 120 ml of refluxing 6 N HCl for 6 hr. After chilling overnight, the hydrochloride salt of the product which formed was removed by filtration. The crystalline solid was dissolved in a minimum amount of water, and the solution was adjusted to pH 7 by addition of 1 N NaOH to form a precipitate. There was recovered 3.07 g (73.8%) of product. Recrystallization from hot water gave an analytical sample, mp 245-246 C (dec). The R_f values of this product in *n*-butanol-acetic acid-water (3:1:1) and 65% pyridine were 0.59 and 0.73, respectively. Anal. Calcd. for $C_{10}H_{12}INO_3$: C, 37.40; H, 3.77; N, 4.36. Found: C, 37.73; H, 3.64; N, 4.39.

2-Iodotyrosine (4) Method A.

To a slurry of 3.0 g (0.009 mol) of 2-iodo-4-methoxyphenylalanine in 25 ml of methylene chloride was added dropwise 70.2 ml (0.028 mol) of 10% BBr_3 in methylene chloride at Dry Ice-isopropanol temperature. The mixture was then

allowed to come to 25 C and stirred for 36 hr. Water was added with vigorous stirring, and the methylene chloride was removed *in vacuo*. The solution was adjusted to pH 7 by addition of 1 N NaOH, and then chilled overnight to yield 1.2 g (42.2%) of product. An analytical sample, mp 268-270 C (dec) was obtained by recrystallization from hot water. The R_f values of this product in *n*-butanol-acetic acid-water (3:1:1) and 65% pyridine were 0.51 and 0.76, respectively. The IR spectrum showed major absorption bands at 3500 (broad), 3050 (broad), 1605, 1575, 1520, 1490, 1450, and 1250 cm^{-1}; ^1H NMR ($DCl-D_2O$) τ 2.55-3.10 (m, 3, ArH), 5.60 (d of d, 1, J = 8.6 Hz and J = 7.7 Hz, $-CH_2-CH-$), 6.72 (d of d, 2, J=8.6 Hz and J = 7.7 Hz, $Ar-CH_2-$). Anal. Calcd. for $C_9H_{10}INO_3 \cdot H_2O$: C, 33.54; H, 3.69; N, 4.04.

Method B.

A solution of 1.0 g (0.0022 mol) of ethyl α-acetamido -α-(2-iodo-4-methoxybenzyl) malonate and 10 ml conc. HBr was heated to reflux for 24 hr. The formation of iodine crystals in the reflux condenser was observed within 1 hr. A paper chromatogram of the reaction mixture in *n*-butanol-acetic acid-water (3:1:1) showed the presence of tyrosine (R_f 0.35) and 2-iodotyrosine (R_f 0.51). The volume of solution was reduced to dryness *in vacuo* and the residue was dissolved in absolute ethanol and then neutralized by the addition of 10% aqueous NaOH to give 160 mg (22.8%) of product. This product was unequivocally identified by melting point, R_f values, and IR spectral analysis as 2-iodotyrosine with an authentic sample prepared by Method A.

Hydrogenolysis of 2-Iodotyrosine.

A 100 mg sample (0.00031 mol) of 2-iodotyrosine in 10 ml of 50% aqueous methanol was hydrogenated at 3.67 kg/cm^2 of H_2 pressure in the presence of Pd/C catalyst for 3 hr. The catalyst was removed by filtration, and the filtrate was treated with charcoal. The solution was filtered, and the resulting filtrate was adjusted to pH 7 with 10% aqueous NaOH and chilled overnight to give a precipitate. There was recovered 50 mg (89.8%) of solid by filtration. The IR spectrum of the solid was identical to that recorded for DL-tyrosine.

Microbiological Assays.

For *E. coli* (ATCC 9723) a previously described assay procedure (Dunn, *et al.*, 1956) and inorganic salts-glucose medium (Anderson, 1946) was used. For *S. faecalis* (ATCC 8043) and *L. plantarum* (ATCC 8014, *L. arabinosus* 17-5), the procedure and basal medium were the same as previously reported (Ravel, *et al.*, 1954) except 100 μg/ml of calcium pantothenate was included in the vitamin supplement and tyrosine and phenylalanine were omitted from the amino acid medium. The basal medium for *S. faecalis* was further modified by adding 20 μg/ml of glutamine under aseptic conditions.

The 2-iodotyrosine and related compounds were dissolved in sterile water to prepare 1 mg/ml solutions at 25 C. From these solutions, serial dilutions were made to the desired concentrations and added aseptically to the previously autoclaved assay tubes without being heated. After inoculation, the assay tubes with *E. coli* were incubated at 37 C for 16 hr, and those with *S. faecalis* and *L. plantarum* were incubated at 30 C for 24 and 30 hr, respectively.

In all assays the amount of growth was determined spectrophotometrically at 625 mμ with a Bausch & Lomb Spectronic 20 in terms of absorbance readings of the turbid culture medium against a blank of uninoculated medium set at zero absorbance. In all assays, appropriate controls were used, and the results of the minimum inhibitory concentrations of the various compounds tested were shown to be reproducible on repeating the assay several times.

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Al^{26} CONTENT OF THE DALGETY DOWNS METEORITE

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INTRODUCTION

The Dalgety Downs stone has been recognized as a meteorite since 1941. It was not seen to fall and the exact date it was found is not known. It was found 6 mi south of Dalgety Downs in Western Australia. In August 1963, Dr. E. P. Henderson of the Smithsonian Institute, Washington, D.C., and Dr. B. Mason of the American Museum of Natural History, New York, collected about 400 lbs of this meteorite. We are grateful to Dr. Henderson for providing us with a 1.79-kg sample of this meteorite for this study. The surfaces of the samples they collected were extensively weathered; the centers were relatively fresh, although evidence of oxidation was nonetheless obvious. The work of Goel and Kohman (1962) and Suess and Wänke (1962) has shown that a relatively fresh looking meteorite such as the Potter County, Nebraska, stone has been on earth for a few tens of thousands of years ($\geq$ 21,000 yrs). The area in which the Dalgety Downs samples were collected has an annual rainfall of only 10 to 14 in. The particular spot where the samples were located when found was on a slight elevation where drainage appeared good (Henderson, pers. comm.). Since weathering would be expected to be rather slow, it is conceivable that the extensive weathering of Dalgety Downs might be the result of a terrestrial residence time of the order of a million yrs or so. It is of interest, therefore, to attempt to determine whether this meteorite is an old fall or if it is a comparatively recent one. It was with this in mind that Dr. Henderson asked if we could conduct some non-destructive tests to determine the terrestrial age of this sample.

If the terrestrial residence time of the meteorite were very long ($\sim 0.5 \times 10^6$ yrs) then some interesting information could be obtained by determining the Al^{26} content of a sample of the meteorite. Since the Al^{26} contents of stone

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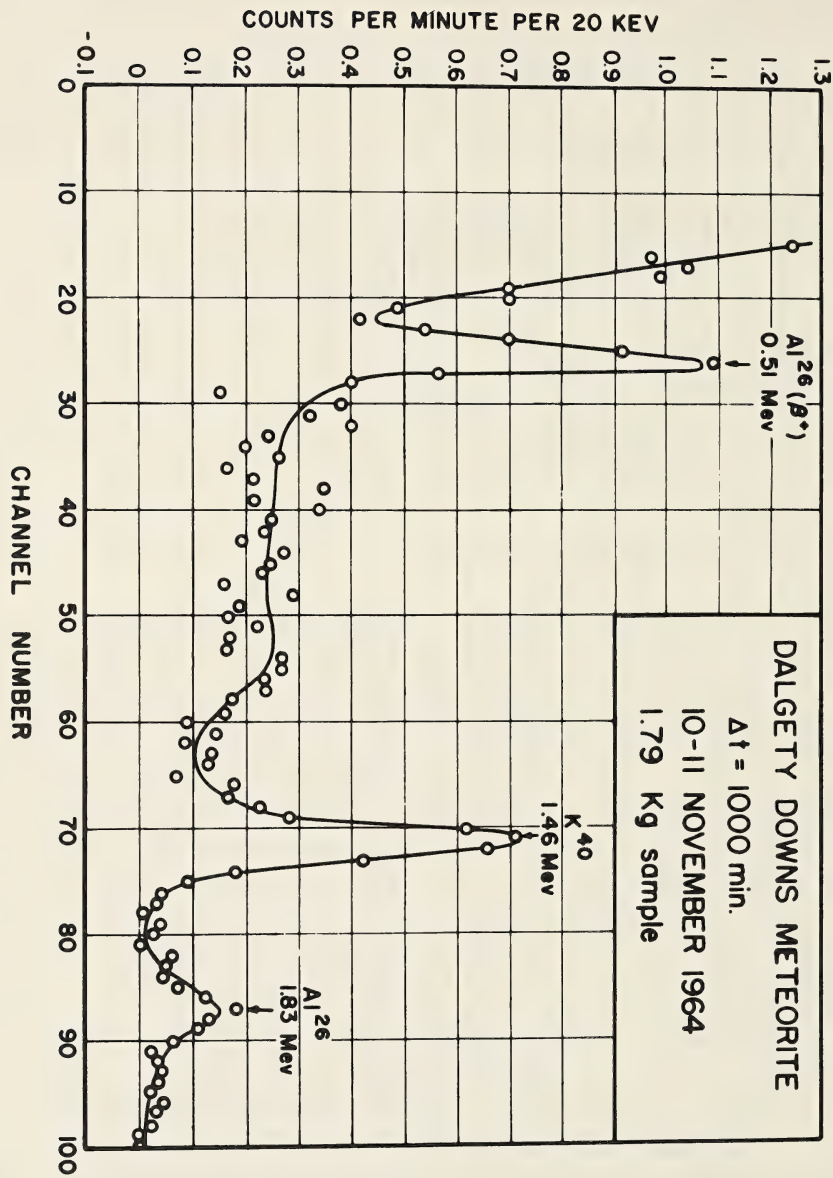

Figure 1. γ-ray spectrum of the Dalgety Downs stone meteorite. Only one of the five 1000-min spectrums is shown; the others are very similar to the one shown.

meteorites (in particular, chondrites) has been shown to be fairly constant at ∿64 dpm/kg (Heymann and Anders, 1967; Fuse and Anders, 1969) it should be possible to ascertain a very long decay time of the order of the Al^{26} half-life. Other factors can cause the Al^{26} to be lower than this average value (Anders, 1962) but a very low value for the Al^{26} of Dalgety Downs would be very suggestive of a long terrestrial age.

The method used here for the measurement of the Al^{26} of meteorites is completely non-destructive and has been previously described by Rowe, *et al.*, (1963) and corrected for peak summing as by Rowe and Clark (1971). These previous studies have shown that only natural K^{40} ($T_{1/2}$ = 1.29 x 10^9 yrs) and cosmic-ray produced Al^{26} ($T_{1/2}$ = 7.4 x 10^5 yrs) are expected in chondritic meteorites with terrestrial ages greater than 10-20 yrs. The results of the measurement of 1.79 kg of Dalgety Downs indicate that this is the case in this study with only the 0.51-MeV positron annihilation photon of Al^{26}, the 1.46-MeV γ-ray of K^{40} and the 1.83-MeV γ-ray of Al^{26} having been detected (Fig. 1). The γ-ray spectrum shown in Fig. 1 was taken using a 3 x 3 in NaI (T1) crystal which feeds pulses into a 400 channel RIDL analyzer and covers the energy range from about 0.2 to 2-MeV.

Since the potassium content of chondritic meteorites has been shown to be fairly constant (0.081%) by Edwards and Urey (1955) and by Rowe, *et al.*, (1963), the 1.46-MeV K^{40} γ-ray photopeak can be used as an internal standard for a good approximation. The 1.83-MeV γ-ray of the Al^{26} was therefore calibrated by comparison with K^{40} γ-ray photopeak. Small corrections are necessary to account for effects due to peak width, photofraction, efficiency and meteorite self-absorption of the 1.46- and 1.83-MeV γ-rays. The photofraction and efficiency corrections were taken from the Monte Carlo calculations of Vegors, *et al.*, (1958). A larger correction for peak summing was necessary and was based on the measurements of Rowe and Clark (1971).

Since the Al^{26} content of Dalgety Downs (60 $\pm$6 dpm/kg) is very similar to the average chondritic value (64 $\pm$10 dpm/kg), it appears extremely unlikely that the Dalgety Downs meteorite has a terrestrial residence time longer than $\sim$100,000 yrs and was probably much less.

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SURVIVAL AND ABUNDANCE OF INTRODUCED FISHES NEAR SAN ANTONIO, TEXAS

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ABSTRACT

Introduced fishes comprise a substantial fraction of the fish fauna in 2 Bexar County waters: 35% in upper San Antonio River and 43% in Braunig Reservoir. Four species (*Astyanax mexicanus, Poecilia latipinna, P. formosa,* and *Cichlasoma cyanoguttatum*) are south Texas fishes displaced northward. Two species (*Cyprinodon variegatus* and *Menidia beryllina*) are recent introductions from estuarine waters. Four species (*Hypostomus plecostomus, Poecilia reticulata, Sarotherodon mossambicus,* and *S. aureus*) are exotics from South America, the Lesser Antilles, Africa, and Israel, respectively. Although *P. reticulata* is now added to the Texas fish fauna the apparent disappearance of another exotic, *Belonesox belezanus*, leaves the number of exotic poeciliids in Texas unchanged. The abundance of exotics is of substantial concern and care should be exercised to reduce the possibility of additional escapes.

INTRODUCTION

The release of exotics can have a substantial impact on the indigenous biota. Although any taxon may be affected, we have concentrated our efforts on fishes. The number of exotic fishes now available for release is of substantial concern (Lachner, *et al.*, 1970). We consider intracontinental as well as intercontinental transfers as exotics because they also have a potential impact on the native fishes (Hubbs, 1977).

Since released fishes must compete with the indigenous fishes to survive and reproduce, establishment of a breeding population would be most likely if relatively few native fishes occurred in the area (Hubbs, 1972). Two such environments occur in Bexar County: stenothermal springs (Hubbs, 1971) and power

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plant reservoirs (Thompson, *et al.,* 1977). The former are of more concern as those springs have an adapted endemic biota (Minckley and Deacon, 1968) whereas the latter are newly constructed environments.

Two of the major Texas spring runs, San Marcos and San Antonio, seem to have been subjected to release of exotics. Brown (1953) documented the exotic nature of the San Marcos spring run fauna. No such summary has been made for the San Antonio spring run, although Barron (1964) reported the presence of *Hypostomus* (= *Plecostomus*) *plecostomus* and *Belonesox belezanus.* Our studies confirmed the presence of the former but the latter may well have died out. We also discovered the presence of *Sarotherodon mossambicus* and *Poecilia reticulata.*

Thermal reservoirs may have a substantial exotic component in their fauna. For example, Noble, *et al.,* (1975) reported dominance by *Sarotherodon* (= *Tilapia*) *aureus* in Trinidad Reservoir, Texas. A similar abundance of blue tilapia prevails in Braunig Reservoir. They are supplemented by substantial numbers of *Cyprinodon variegatus* and *Menidia beryllina* as well as some *Poecilia formosa,* all coastal fishes seemingly recently introduced.

COLLECTION LOCALITIES

The San Antonio River was sampled November 18, 1977. Our sampling was from 60 m north of Hildebrand Avenue in the headwaters to 30 m south of the now abandoned low water crossing connecting River Road and Avenue A (Fig. 1).

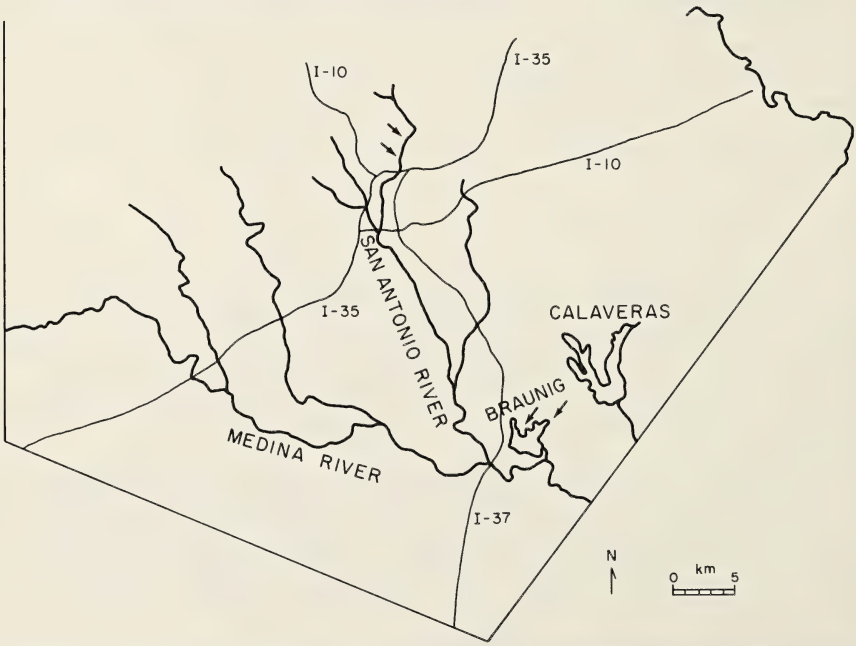

Figure 1. Sketch map of southern Bexar County showing locations of collection sites.

Measured along the main river channel, these 2 points are 3 km apart. We collected extensively at the upper and lower locations as well as between the Witte Museum and Joske Pavilion, near the horse crossing, and from Mulberry Avenue south for about 150 m. In addition to the above main channel sites, we sampled the 2 ditches draining the San Antonio Zoo's well-fed stream system into the San Antonio River, the channel connecting bends in the River just east of the Park Office, and a pool formed by damming a spring-fed headstream just north of the Park Office. We used a variety of seines from 2-10 m and mesh sizes as small as 2 mm.

Braunig Lake was sampled September 29, 1977. Our sampling was by beach seine at the power plant outlet and from the northeastern cove. Our results in many ways were similar to those obtained in a Texas Parks and Wildlife cove rotenone sample taken earlier that week.

San Antonio River

Our San Antonio River sample included 17 fish species, 11 (*Notropis lutrensis, N. amabilis, N. volucellus, Ictalurus natalis, Gambusia affinis, Micropterus salmoides, Lepomis gulosus, L. cyanellus, L. megalotis, L. punctatus,* and *L. macrochirus*) likely to be native and 6 (*Astyanax mexicanus, Hypostomus plecostomus, Poecilia reticulata, P. latipinna, Cichlasoma cyanoguttatum,* and *Sarotherodon mossambicus*) considered to be exotics. These 6 will be discussed below.

Astyanax mexicanus is an abundant midwater small fish. We encountered numerous schools throughout the area sampled. Brown (1953) reported the Mexican tetra (as *Astyanax fasciatus mexicanus*) was introduced into the Guadalupe Basin between 1908 and 1930 with the first San Antonio River record in 1940. Clearly, the fish has long been established. Presumably, the stocks came from those native to the Rio Grande or Nueces River.

Hypostomus plecostomus is an abundant bottom fish. We preserved 50 individuals and could have obtained many more. Our specimens ranged from 13-238 mm SL. All evidence suggests a long breeding season-even distribution of fish sizes, small to breeding adults, including a 182 mm female with 716 yolked eggs 3.25 mm in diameter. Our findings agree with those of Barron (1964) who reported that this exotic (listed as *Plecostomus* sp.) breeds in the San Antonio River. Our sample contained 13 mature or maturing males and 8 females. Clearly, the largest individuals were males (8 were 199 mm or longer), while the longest female was the 182 mm ripe individual. Five "maturing" males were 130-180 mm and 7 "maturing" females were 116-181 mm. Large *Hypostomus* were taken from the swiftest waters, often under large boulders. Some were seined from the tail race downstream lip of a low water crossing. Young and juveniles were often taken from heavy vegetation in slow to moderate current. Seining the armadillo del rio [a name we propose for the common name as it complies with recommendations 10, 11, and 14 of Bailey, *et al.*, (1970)] was best done by techniques normally associated with the capture of darters--adults where one would expect

Percina caprodes and young in a location similar to that inhabited by *Etheostoma fonticola*. Juveniles and adults were observed moving rapidly in the current in a manner that commonly is done by darters. It is unlikely that competition for food is involved as our specimens of *Hypostomus* had long guts filled with algae and other vegetal material whereas darters are primarily arthropod feeders. If the armadillo del rio have displaced a native fish, that displacement is most likely to involve the stoneroller (*Campostoma anomalum*) or roundnose minnow (*Dionda episcopa*), both of which are commonly very abundant in central Texas spring runs but were absent from our samples.

Poecilia reticulata is found abundantly in the north ditch draining the San Antonio Zoo. We preserved more than 100 young to breeding adult guppies and several more were maintained in a live sample. Most of the large females were gravid. Males were very colorful and on occasion females had modest coloration. Clearly, the fish are descended from aquarium stocks selected for bright colors.

Guppies have not previously been recorded as residents in natural waters in Texas. Edwards (1976) reported a sparse population in Waller Creek in Austin that failed to survive the following winter. The San Antonio stocks exist in stenothermal well waters similar to spring waters in which released guppies have survived elsewhere in the United States (Deacon, *et al.*, 1964). Although guppies were not collected from the San Antonio River itself, it is highly probable that the population will perpetuate and disperse to the river following selection for wild-type genes. Regardless of their spread, the population we sampled seemed to be surviving under somewhat natural conditions and can be construed to be an addition to the existing fish fauna of the state. Guppy abundance may have been enhanced by the relative scarcity of predacious centrarchids in that ditch. The absence of *Belonesox belezanus* in our samples suggests the failure of that release reported by Barron (1964), leaving the number of poeciliids in Texas unchanged.

Poecilia latipinna is abundant, especially in shallow waters with minimal current. Brown (1953) had reported a 1939 escape of sailfin mollies (as *Molliensia latipinna*) from a Florida stock into the San Antonio River. The first collections from the Guadalupe River System were in 1941. Brown also reported releases of a Louisiana stock and presumably releases from native Texas stocks may also have occurred. It is unknown which race (or races) now is represented in the abundant populations currently found in the Guadalupe System.

Cichlasoma cyanoguttatum is an abundant quiet water fish in much of the upper San Antonio River. The native Texas range of the Rio Grande cichlid was limited to the Nueces and Rio Grande systems (Brown, 1953). He reported that Rio Grande cichlids (as *Herichthys cyanoguttatus*) were raised in the National Fish Hatchery in San Marcos between 1928 and 1941. Presumably, the San Antonio River stocks were released in 1929 or shortly thereafter. The stocks came from near Mission, Texas.

Sarotherodon mossambicus is found in small numbers in the south ditch draining the San Antonio Zoo near the train station. In addition to the fish in our collections, we observed several decomposing adults in that region. Apparently they are most abundant in the deepest water (= hardest to sample with the seines we used). They have also been observed to be very abundant in canals in the adjacent San Antonio Zoo, especially the internal ditches used for duck and water bird exhibits. These locations empty into the south drainage ditch. The Mossambique tilapia was reported (as *Tilapia mossambica*) by Hubbs (1961) to have been released in the San Marcos and San Antonio springs but the source was not mentioned. Atz (1954) mentioned its extensive dissemination as an aquarium fish and the release was presumably from this provenance. The impact of the Mossambique tilapia is expected to be on the Rio Grande cichlid which also is not native to the San Antonio River.

Braunig Lake

Our Braunig Lake sample included 14 species, 7 (*Dorosoma cepedianum, D. petenense, Pimephales vigilax, Gambusia affinis, Lepomis cyanellus, L. megalotis,* and *L. macrochirus*) likely to be native, 1 (*Notemigonus crysoleucas*) possibly native, and 6 (*Cyprinodon variegatus, Poecilia latipinna, P. formosa, Menidia beryllina, Cichlasoma cyanoguttatum,* and *Sarotherodon aureus*) considered to be exotics. Four of these exotics (except *P. latipinna* and *C. cyanoguttatum* which are discussed in the San Antonio River segment of this report) will be discussed below.

Cyprinodon variegatus is found in shallow water over soft sand and mud substrates with minimal vegetation throughout Braunig Reservoir. We also observed numerous individuals breeding in shallow waters at the southwestern corner of the reservoir. The natural range of this species is in estuarine waters throughout Texas and adjacent areas. Sheepshead minnows recently have turned up at several inland localities. It is uncertain whether these Braunig Reservoir fish reflect a similar recent introduction or whether this population was introduced earlier and is the source of the existing west Texas populations (Stevenson and Buchanan, 1973; Hubbs, *et al.,* 1978). We do not expect this release will have the substantial deleterious impact that has been observed for those west Texas releases.

Poecilia formosa occurs in sparse numbers in the northeastern cove. This is the first record of the Amazon molly in Bexar County. The natural range in Texas seems limited to the lower Rio Grande tributaries and distributaries south of Raymondville, Texas. Martin (1964) documented other apparent releases (as *Molliensia formosa*) that seem to have increased the geographic range of this fish. It seems likely that Amazon mollies were only recently released in Braunig Reservoir. The heated temperatures should be favorable to the abundance and spread of this gynogenetic fish (Hubbs, 1964) and their abundance should be monitored to determine rates of population increase and/or clonal diversity.

Presumably the fish accompanied a game fish release. Lula Sams State Fish Hatchery would be a reasonable location for the stock source, as Amazon mollies are abundant in many hatchery ponds there. The impact of increased numbers will be primarily at the expense of its sexual host, *P. latipinna*, which is also exotic.

Menidia beryllina is found on sandy beaches in Braunig Reservoir. As with the sheepshead minnows, the natural range of the tidewater silversides is estuaries in Texas and adjacent areas. Tilton and White (1964) have documented their abundance inland following reservoir construction. Thus, it is not surprising to find them in another reservoir. Tidewater silversides seem able to spread far from reservoirs and have been found more than 100 km from the expected reservoir release site (Hubbs, *et al.*, 1977).

Sarotherodon aureus is present in large numbers throughout the reservoir. It may well be the dominant fish in Braunig Reservoir--even out competing gizzard shad. Noble, *et al.*, (1975) have reported the impact of a population explosion in Trinidad Reservoir. Fortunately, massive mortalities (and possible extinction) occurred when that power plant was under repair during cold weather. It is unfortunate that the stock seems to have been perpetuated elsewhere (Canadian River, Oklahoma, near a power plant, J. Pigg, personal communication, 1977). The exact source of the Braunig Lake and other Texas stocks (Hubbs, 1976) is uncertain but those releases should be restricted as much as possible to avoid problems similar to those that developed in Trinidad Reservoir. Edwards has repeatedly collected blue tilipia of various sizes from shallow weedy areas in Canyon Reservoir on the Guadalupe River. The Canyon Reservoir population may have adapted to central Texas thermal conditions or submerged springs emptying into the reservoir may provide a thermal refugium in winter.

DISCUSSION

Exotic fishes currently abound in the vicinity of San Antonio, Texas. In our 2 samples exotics accounted for 35 and 43% of the species collected. Some such as *Cichlasoma cyanoguttatum, Poecilia latipinna,* and *Astyanax mexicanus* also occurred more than 3 decades ago. The impact of those releases is uncertain as the prior fauna was too little known. Recent releases such as *Poecilia formosa, Sarotherodon aureus,* and *S. mossambicus* are likely to be impacting previously released exotics. Other "new" releases may affect native fishes but the absence of a substantial prior population study makes any conclusions quite tentative. We are most concerned about the impact of releases into the San Antonio spring run as the negative impact is likely to include depleting the abundance of native fishes adapted to that stream.

We recovered an equivalent abundance of exotics in Braunig Reservoir but if the exotics remain restricted within the confines of that artificial impoundment (current and thermal), environmental problems may be manageable. Escape into natural waters and adaptation to environmental factors could produce an added

threat to the native fauna of Bexar County. The estuarine fishes (*Menidia beryllina, Poecilia formosa, P. latipinna, Cyprinodon variegatus*) may have accompanied marine fishes released for sport fishing purposes. For example, red drum (*Sciaenops ocellata*) are taken from Braunig Lake by sport fishermen. It is likely that red drum do not reproduce in Braunig and their presence there depends upon periodic stocking of young individuals. We urge extreme care to accompany such transport to avoid unintentional releases.

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A STUDY OF PHYTOPLANKTON PRIMARY PRODUCTIVITY AND NUTRIENT CONCENTRATIONS IN LIVINGSTON RESERVOIR, TEXAS

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ABSTRACT

A 7-month study of phytoplankton primary productivity using a carbon-14 technique was conducted at Livingston Reservoir, Texas. The reservoir was found to be eutrophic with hypereutrophic conditions in the riverine portion. The lacustrine region was most productive during the spring and early fall, but the riverine region had the highest rates during the summer. Regression analysis indicated turbidity was a major factor limiting production rates. Water hyacinth populations appear to be causing a significant reduction in nitrate and phosphate concentrations in the riverine portion of the reservoir.

INTRODUCTION

An assessment of primary productivity is essential in management of inland waters. Although extensive chemical water quality data on Livingston Reservoir can be found in U.S.G.S. reports (1975), Texas Water Quality Board publications (1973) and Trinity River Authority studies (1976), no primary productivity data for the reservoir have been found in the literature. The purpose of this study was to determine the rates of phytoplankton primary productivity and its relationship with certain physico-chemical parameters at Livingston Reservoir from April 5, 1975 through October 25, 1975.

DESCRIPTION OF THE RESERVOIR

Livingston Reservoir is located on the Trinity River in Polk and San Jacinto Counties, 9.6 km southwest of Livingston, Texas. Completed in 1968, the reservoir is owned jointly by the Trinity River Authority and the city of Houston

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to supply municipal, industrial and irrigation water for the Houston area. It is also widely known as a recreational area. The conservation storage capacity is 1,750,000 acre ft ($2.16 \times 10^9 M^3$) with a maximum capacity of 3,045,000 acre ft ($3.75 \times 10^9 M^3$). The reservoir is approximately 48 km in length and has a surface area of 33,428 ha at normal operating level, with a mean depth of 6.27 m. The reservoir receives most of its water from the Trinity River, but tributaries such as Harmon Creek, White Rock Creek, Kickapoo Creek, and others provide inflow during periods of high run-off. Land use around the reservoir which may influence water quality include paper and lumber interests, cropland and livestock operations, but perhaps the most important uses are recreational facilities, resort areas, retirement and "second-home communities."

METHODS

Ten collecting stations were selected to represent the upper, middle and lower regions of the reservoir. Stations 1 through 5 were located in the more lacustrine region of the reservoir with Station 4 located in the bay formed by Kickapoo Creek. Stations 6 through 10 were located in the more riverine portion of the reservoir, with Stations 6, 7 and 8 reflecting the possible influence of White Rock Creek and Stations 8, 9 and 10 reflecting the influence of Harmon Creek (Fig. 1).

Physico-Chemical Methods

Water samples were collected on 11 separate dates concurrently with productivity determinations. All water samples were collected at the 0.5 m depth in clean, plastic containers. Samples used for subsequent laboratory analyses were immediately placed on ice and all chemical determinations were begun within 6 hrs after collection. Dissolved oxygen and temperature were determined at the 0.5 m depth with a YSI Oxygen Meter, Model 54. Free carbon dioxide, carbonate, and bicarbonate concentrations were determined in the field using titrametric techniques described in Standard Methods (Amer. Public Health Assoc., 1971). Turbidity was determined using a Hach Turbidimeter, Model 2100A, while pH was determined in the laboratory using a Corning pH Meter, Model 12. Secchi disc transparency was done according to Lind (1974).

An *in vivo* measurement for chlorophyll *a* was made on each water sample according to methods suggested by Lorenzen (1966) and Yentsch and Menzel (1963) using a filter fluorometer, Turner Model 110. Chlorophyll *a* concentrations are reported in $\mu g/l$ of reservoir water. Light extinction coefficients were computed according to Lind (1974).

Nitrate nitrogen, nitrite nitrogen, ammonia nitrogen, and orthophosphate concentrations in water samples were determined using colorimetric procedures reported in Standard Methods (Amer. Public Health Assoc., 1971). Optical density at each specified wavelength was made using a Bausch and Lomb spectrophotometer, Model 70. Orthophosphate was determined using the stannous chloride

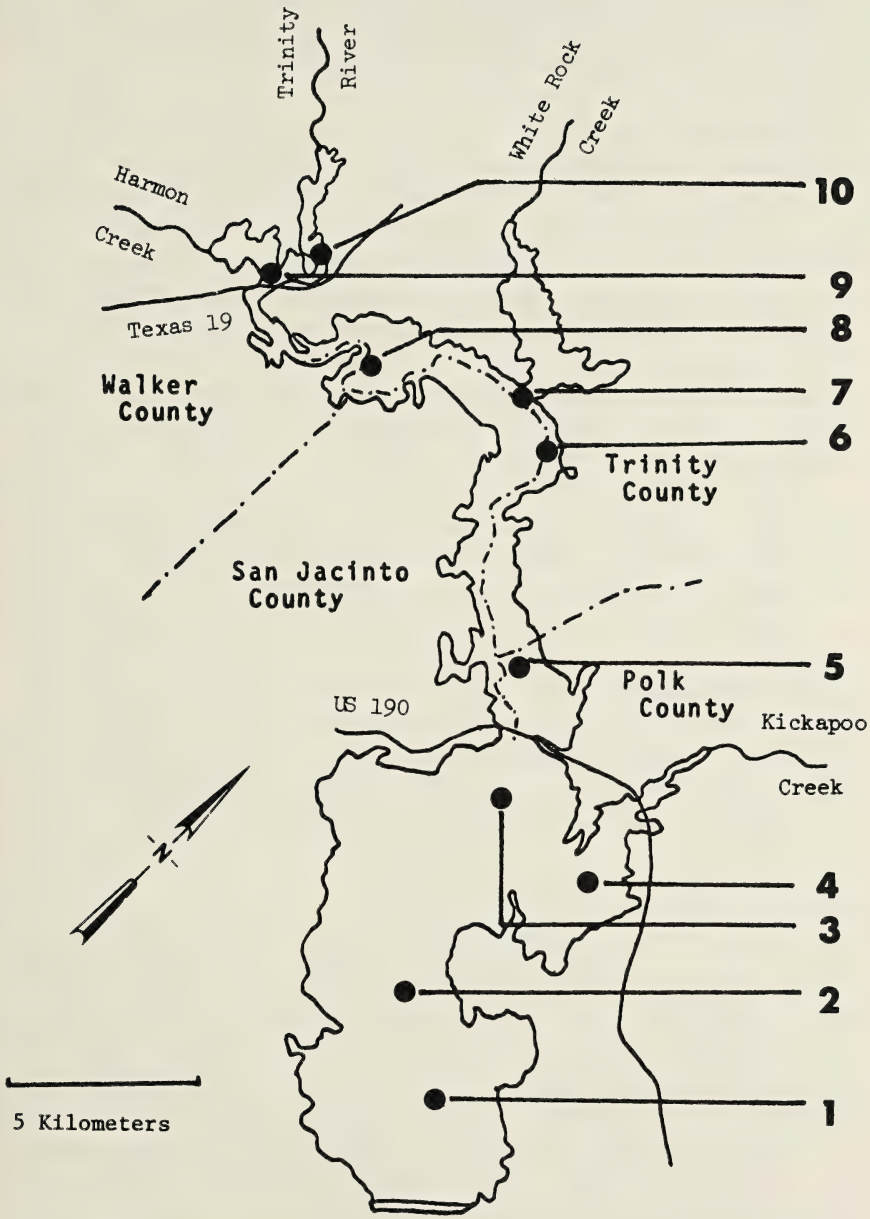

Figure 1. Diagram of the Livingston Reservoir including location of the sampling stations.

method, nitrate nitrogen was determined by the cadmium reduction method, nitrite nitrogen by the diazotization method, and ammonia nitrogen by the Nessler method.

Incident light values recorded on the dates of primary productivity determinations were obtained from the Texas A&M University Weather Station located at College Station, Texas, and are reported in Langleys/day.

Primary Productivity Methods

The carbon-14 method used in this study was first employed by Steeman-Nielsen (1951-1952). Modifications of this technique reported by Strickland (1966) and Vollenweider (1969) have been implemented. Primary productivity estimates were conducted 11 times during the study period, involving 211 light bottle and 109 dark bottle analyses. Three water samples were collected at the 0.5 m depth at each station on each collection date. Because of the relatively shallow compensation depth at each station, only the surface m^3 of water was considered. Two samples were collected in clear 250 ml bottles and 1 sample collected in a 250 ml bottle darkened with plastic tape and aluminum foil to insure no light would penetrate. Five μci of carbon-14 as $NaH^{14}CO_3$ were added to each bottle which were incubated *in situ* for 6 hrs at the 0.5 m depth. All samples were incubated during the same general time period beginning in the early morning and ending in the afternoon. Subsequent to incubation, samples were filtered through Millipore filters with 0.45 ± 0.02 μ pores. Radioactivity of each filter was determined by a gas flow proportional counter, Nuclear Chicago, Model 1042, automatic sample changer, along with a Model 8703 decade scaler and lister. Equations for calculating mg of carbon assimilated are reported by Vollenweider (1969), and values are reported as mg of carbon assimilated/surface m^3 of water/hr ($mgC/m^3/hr$). Primary productivity estimates were made at all 10 stations on the same day, thus on each collecting date production determinations were made under similar meteorological conditions.

RESULTS AND DISCUSSION

The results of physico-chemical analyses and productivity determinations are given in Table 1. The mean rate of phytoplankton productivity for the reservoir was 70.9 $mgC/m^3/hr$, with a range of 358.6 $mgC/m^3/hr$ at Station 8 in July to 0.8 $mgC/m^3/hr$ at Station 10 in May. Station 7 had the highest mean rate of productivity with 91.9 $mgC/m^3/hr$ and Station 10 the lowest at 52.9 $mgC/m^3/hr$. When the primary productivity results are compared with other studies, Livingston Reservoir should be generally classed as a eutrophic body of water with hypereutrophic conditions existing in the more riverine portions (Stations 6 - 10).

The seasonal effect on the average rate of phytoplankton production in the reservoir was pronounced, with the mean rate increasing from 36.0 $mgC/m^3/hr$ in April, to a maximum of 141.4 $mgC/m^3/hr$ in August, then declining to a low of 12.1 $mgC/m^3/hr$ in October (Fig. 2). Two interrelated factors accounted for this trend. First, the incident light recorded on the days when productivity

values were obtained increased from a daily total of 233 Langleys in April to a maximum of 638 Langleys in August, then declined to 44 Langleys in October. Second, the flow rates declined in the Trinity River during the summer months, producing relatively low turbidity in the riverine region, a part of the reservoir where large concentrations of nitrate and phosphate ions were typically found. These circumstances resulted in the production of a large phytoplankton standing crop and high rates of production during the summer. The most productive

TABLE 1

The Mean Value ($\bar{x}$) and Standard Deviation (σ) of Physical-Chemical and Productivity Determinations at Each Collecting Station on Livingston Reservoir from April 6, 1975 to October 25, 1975

Station	NH_3-N (PPM)		NO_3-N (PPM)		NO_2-N (PPM)		PO_4 (PPM)	
	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
1	.61	.20	.20	.12	.008	.012	.36	.12
2	.63	.21	.24	.17	.008	.012	.18	.13
3	.93	.38	.33	.25	.011	.012	.48	.14
4	.77	.28	.15	.06	.011	.010	.35	.12
5	.90	.38	.36	.29	.006	.010	.53	.14
6	1.00	.45	.43	.35	.006	.007	.54	.17
7	1.02	.39	.40	.33	.005	.008	.50	.17
8	1.14	.42	.92	.47	.021	.032	1.13	.77
9	1.05	.34	.84	.60	.026	.049	1.17	1.11
10	1.17	.48	.98	.46	.029	.046	1.29	1.01

Station	pH		Turbidity (NTU)		Chlorophyll a (μg/l)		Light Extinction Coefficient
	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$
1	8.54	.40	3.63	1.02	18.99	11.20	1.60
2	8.40	.49	3.90	1.59	21.47	19.76	1.60
3	8.43	.54	11.00	10.23	27.38	18.55	3.00
4	8.58	.56	5.36	1.43	28.41	13.04	2.10
5	8.50	.51	15.09	10.94	25.10	10.07	3.20
6	8.37	.62	17.36	12.92	25.50	15.40	3.50
7	8.44	.60	16.45	11.21	31.18	17.84	3.50
8	8.22	.60	25.18	15.52	23.28	23.79	4.70
9	8.26	.51	21.63	13.66	22.74	20.08	4.00
10	8.11	.58	32.90	17.87	22.21	26.69	5.20

(cont.)

Table 1 (Continued).

Station	O_2 (PPM)		CO_2 (PPM)		$^{-}HCO_3$ (PPM)		$^{=}CO_3$ (PPM)	
	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
1	7.15	2.32	.18	.60	85.45	8.09	29.81	12.31
2	7.54	1.95	.40	.84	88.90	11.78	20.00	14.90
3	6.34	2.92	.81	1.60	93.90	15.26	16.81	14.63
4	7.30	2.57	.18	.60	83.45	8.89	25.36	13.72
5	6.47	3.06	.63	1.56	93.36	13.14	16.90	13.39
6	6.03	2.38	1.18	1.60	103.27	15.06	12.36	14.90
7	6.10	2.53	1.00	1.18	95.00	20.23	12.72	15.85
8	5.20	1.91	2.00	2.19	115.00	19.70	7.90	11.49
9	5.23	1.75	1.54	1.69	110.45	20.14	6.54	8.35
10	5.16	1.89	2.72	2.37	114.18	18.72	7.27	11.32

Station	Temp. (C)		Secchi (cm)		Productivity ($mgC/m^3/hr$)	Depth (m)	
	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$
1	25.36	4.45	104.90	14.41	54.40	23.58	12
2	25.00	4.69	103.20	11.76	53.55	35.30	14
3	25.54	4.94	55.54	17.46	83.75	56.93	6
4	25.36	4.82	79.45	10.45	84.38	52.53	5
5	25.72	4.62	51.54	14.60	71.97	41.45	6
6	25.63	4.75	47.72	15.72	82.03	71.52	6
7	25.45	5.14	47.54	13.57	91.94	82.95	5
8	25.00	5.51	36.09	14.38	71.68	91.01	5
9	25.72	5.81	42.18	19.07	59.69	52.64	4
10	25.00	5.49	32.54	14.18	52.97	70.22	5

region of the reservoir also changed with the seasons (Fig. 2). In April, May and June, Station 3 and occasionally Station 4 were the most productive locations, both in the lacustrine segment. During the summer months, the most productive station moved progressively upstream into the riverine portion of the reservoir. On July 28, Station 8 was the most productive, and on August 30, the region encompassed by Stations 8, 9 and 10 had the highest rates. Increased transparency of the water column in the reverine region during summer when flow rates in the Trinity River were low account for this trend. During the study period, flow rates decreased in the river near the head-waters of the reservoir from a high of 26,300 CFS in April, to a low of 679 CFS in September (U.S. Geol. Surv., 1975). The mean retention time for the reservoir during 1975 was 2.84 months (K.C. Rudy, Trinity River Authority, Pers. Comm).

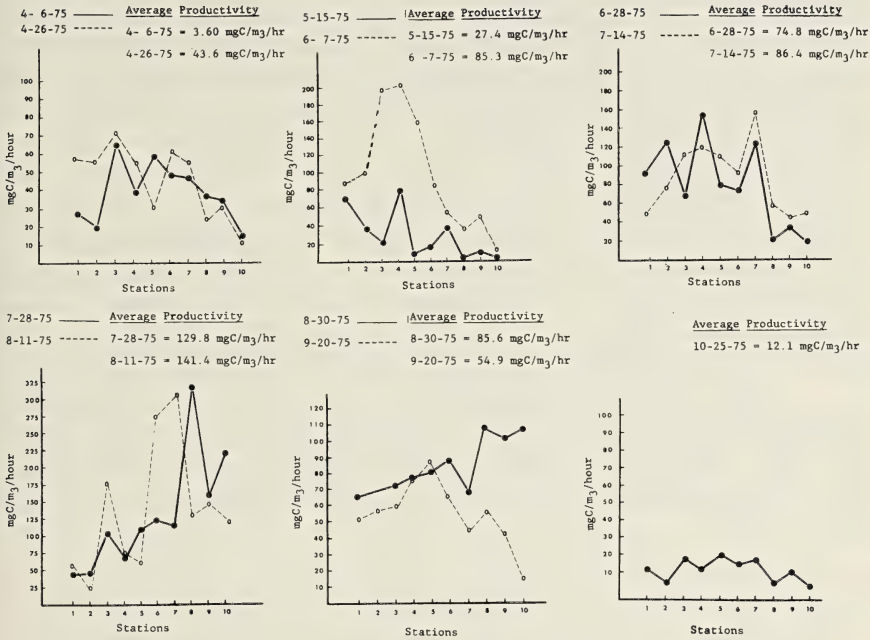

Figure 2. Phytoplankton primary productivity rates at collecting stations on Livingston Reservoir from April 6 - October 25, 1975.

The importance of transparency as a water quality parameter segregating the lacustrine and riverine regions of the reservoir was emphasized by a Stepwise Discriminate Analysis. When all the physico-chemical parameters considered in this study, along with productivity were entered into computer program BMDO7M-Stepwise Discriminate Analysis Secchi disc transparency was found to be the most important parameter identifying those 2 regions.

Interestingly, the average rate of phytoplankton production during the study period was the same for the lacustrine and riverine segments, 71 $mgC/m^3/hr$, however, production rates are correlated with a different combination of environmental factors in the 2 regions of the reservoir. Productivity in the riverine segment was inversely correlated with levels of nitrate ($r = -.72$), ammonia ($r = -.71$), phosphate ($r = -.38$) and turbidity ($r = -.52$), while positively correlated with chlorophyll *a* concentrations ($r = .92$), pH ($r = .87$), and incident light values ($r = .66$). Nitrogen and phosphorus values were significantly higher in the riverine portion and one would expect this to correlate with high rates of production. Higher turbidity values in that segment inhibit the process. When the river flow rates were relatively high, turbidity, ammonia nitrogen, nitrate nitrogen, and phosphate concentrations increased. Chlorophyll *a* concentrations had the best

correlation with production rates in the riverine area, while incident light had a somewhat weaker correlation. It should be pointed out that increased incident light values correspond to the times when turbidity levels were relatively low in the riverine area. The regression equation in Table 2 incorporates 5 variables in predicting productivity in the riverine area and those 5 parameters account for 88% of the variation in production rates while giving a 0.94 multiple correlation coefficient.

In the lacustrine area, turbidity, nitrogen and phosphorus values were weakly correlated with productivity (turbidity r = -.15; ammonia r = .02; nitrate r = -.23; phosphate r = -.18), while phytoplankton standing crop as reflected by chlorophyll *a* concentrations, pH, and incident light values were more strongly correlated (chlorophyll r = .78; pH r = .61; Langleys of light r = .77). These data indicate that turbidity is not an important factor limiting production in this region of the reservoir, and that the rates are primarily dependent upon phytoplankton standing crop and the amount of light impinging on the water surface. The multiple regression equation (Table 3) indicates that chlorophyll *a* concentrations and incident light values could be used to predict production rates in the lacustrine region (r = .96) and those 2 variables account for 92.6% of the variation in productivity.

TABLE 2

Results of Multiple Regression Analysis of Primary Productivity Rates and Selected Water Quality Parameters in the Riverine Portion of Livingston Reservoir

Regression Equation	$Y = B_0 + B_1 X_1 + B_2 X_2 \ldots Bp\ Xp$
	Multiple R = 0.94
	R-Square = 0.88
Variables	Regression Coefficients
X_1 = Chlorophyll *a*	B_1 = 1.60
X_2 = pH	B_2 = 18.20
X_3 = Nitrate	B_3 = 20.24
X_4 = Langleys/day	B_4 = 2.09
X_5 = Turbidity	B_5 = -1.38
Y = Productivity	B_0 = (intercept) = -179.2

Other characteristics of the reservoir indicate eutrophic conditions. An average chlorophyll *a* concentration of 24.6 μg/l with a maximum of 69.4 μg/l clearly reflect a very large phytoplankton standing crop. Michalski and Conroy (1972) state that concentrations of phytoplankton chlorophyll *a* in excess of 10 μg/l reflect nuisance algal crops. A mean Secchi disc depth of 0.59 m, a mean light extinction coefficient of 2.82 as well as large nitrogen and phosphorus concentrations all reflect a eutrophic reservoir.

TABLE 3

Results of Multiple Regression Analysis of Primary Productivity Rates and Selected Water Quality Parameters in the Lacustrine Portion of Livingston Reservoir

Regression Equation	$Y = B_0 + B_1 X_1 + B_2 X_2 + \ldots Bp Xp$
	Multiple R = 0.96
	R-Square = 0.92
Variables	**Regression Coefficients**
X_1 = Chlorophyll *a*	$B_1 = 1.63$
X_2 = Langleys/day	$B_2 = 1.42$
Y = Productivity	B_0 (intercept) = −16.03

White Rock and Harmon Creeks, large tributaries in the riverine portions of the reservoir, appear to have little effect on the eutrophic conditions in that region. An analysis of variance (ANOVA) was used to detect significant differences in nitrate and phosphate concentrations as well as rates of phytoplankton productivity at Stations 5 – 10. The analysis revealed that there was a significant difference at the 95% confidence level in nitrate and phosphate concentrations among these stations, but there was no significant difference in productivity. Duncan's Multiple Range analysis (Table 4) indicates that Stations 8, 9 and 10 had significantly higher nitrate concentrations than did Stations 5, 6 and 7. However,

TABLE 4

Results of Analysis of Variance and Duncan's New Multiple Range Test of Nitrate, Phosphate Concentrations and Primary Productivity at Stations 5 – 10. Horizontal Lines Connecting Stations Indicate No Significant Difference for that Parameter. Mean Concentrations and Productivity Values are Indicated at Each Station.

STATIONS						
7	5	6	8	9	10	
.501	.536	.548	1.13			
		.548	1.13	1.17		Phosphate
			1.13	1.17	1.29	F = 3.0619[1]
5	6	7	8	9	10	
.365	.437	.400				Nitrate
			.926	.845	.982	F = 4.7827[1]
5	6	7	8	9	10	
71.900	82.000	91.900	71.600	59.700	52.900	Productivity F = 0.4495

[1] Significant at 95% confidence level.

there was no significant difference in nitrate levels among Stations 5, 6 and 7 or among Stations 8, 9 and 10. The phosphate concentrations were not significantly different among Stations 5, 6, 7 and 8, but the concentrations at these locations were significantly lower than at Stations 9 and 10. It should be noted that Station 7 (near White Rock Creek) and Station 9 (near Harmon Creek) did not have significantly higher nutrient concentrations or production rates than the corresponding upstream station, hence one can conclude that those 2 tributaries contribute little to the eutrophication of the reservoir. While not statistically significant, Station 7 occasionally had higher production rates than the upstream station, probably because the water from White Rock Creek was less turbid than the reservoir thus resulting in increased transparency in that general region of the reservoir.

The reduction in nitrate and phosphate concentrations between Station 10 in the riverine region and Station 5 in the lacustrine segment (Fig. 1) may have been due to water hyacinth (*Eichornia crassipes*), which almost covered the reservoir for a distance of 8 km between Stations 8 and 6. The area is so densely populated with the plant, it is known to local fishermen as "the jungle". The uptake of nutrients by this plant population could have been extensive enough to have caused the reduction reported in this study. Dunigan, *et al.*, (1975) found that water hyacinth, in greenhouse conditions, caused the loss of large amounts of NH_4 and NO_3-N and lesser amounts of PO_4 from water in which the plants were growing. It is interesting to speculate on what impact this mass of vegetation is having on the rates of phytoplankton production in the large downstream portion of the reservoir.

ACKNOWLEDGEMENTS

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AN UNUSUAL COLOR PATTERN IN COUCH'S SPADEFOOT, *SCAPHIOPUS COUCHI* (ANURA: PELOBATIDAE)—Amphibian coloration provides protection from predators and injurious rays of the sun and functions in thermo-regulation. Thus, extreme changes in color pattern may represent changes in genetic information or normal developmental conditions. Because color patterns serve as a taxonomic character, recognition of aberrant color patterns is important in studying the systematics of some groups of amphibians. Color patterns are effected by the distribution and differentiation of pigment cells (Watson, 1970, *Advances in Morphogenesis*, 8:41).

Scaphiopus couchi from the Texas Panhandle are typically greenish-yellow with broad yellow stripes and blotches along the dorsum. The typical habitat for Couch's spadefoot in this area is a short-grass plains where the pattern of dorsal stripes and blotches serves a selective advantage by providing greater concealment in the shadows produced in the grassy habitat (Cott, 1975, New York, Barnes and Noble, 508 p.). Thus, extreme alterations of this pattern of coloration may serve as a selective disadvantage to the toad.

A 6.12 cm male *Scaphiopus couchi* was collected on June 23, 1976, on F.M. 1061, Potter County, Texas, following a light rain. The dorsum of this specimen was almost solid olive-green, broken only by an indistinct v-shaped mark between the eyes, and a faint greenish-yellow stripe below the left eye. The toad was normal in all other "typical" characteristics of *Schaphiopus couchi*.

The toad was brought to the laboratory and kept under varying conditions of light and temperature for 14 days. No color change occurred during this time. It was then sacrificed and the internal organs were examined. The specimen was preserved in the natural history collection, Department of Biology, West Texas State University.

One explanation, based on the dermal chromatophore unit (Bagnara, Taylor, and Hadley, 1968, *J. Cell Biol.*, 38:67), of the unusual color pattern is that the darker, more uniform olive-green coloration in this specimen is due to a greater distribution of melanophores and guanophores, and a reduction of xanthophores in the skin. Thus, the color would be a darker, more uniform green. This could be explained in terms of changes in genetic or developmental conditions, or by a lack of the hormone intermedin, which stimulates xanthophores to expand.

Another explanation is that this specimen may represent a chlorotic condition. Jones (1967, *Diss. Abstr. Int.*, 29:1213) noted that many neotropical frogs (Hylidae, Centrolenidae, and Pseudidae) have a great quantity of biliverdin in bone, soft tissues, and plasma. In pseudids this condition occurs at or after metamorphosis and is due to hemoglobin degradation. However, in hylids, it also occurs prior to metamorphosis. It was also noted that among adults, this condition appears to develop at the end of the breeding season. Jones suggested that high temperatures or a malfunctioning or immature liver is responsible for causing red blood cells to lyse.

The latter seems the most likely explanation to be reported here since dissection revealed that much of the soft tissues of the toad contained excessive quantities of a green pigment. It was also noted that the liver was relatively small and quite possibly could have been immature or malfunctioning. It is unlikely that high temperatures could have caused the condition since the toad was captured during the cool portion of the summer, and environmental temperatures during the year to that point had been below average. It is also unlikely that the condition was due to physiological changes brought about by the end of the breeding season since the toad was captured in the middle of its breeding season. Thus, we suggest that this abnormal coloration was due to a chlorotic condition brought about by possible liver malfunction.—*Flavius C. Killebrew, Department of Biology, West Texas State University, Canyon 79016, and Raymond C. Stone, Jr., Department of Zoology, University of Tennessee, Knoxville, Tennessee.*

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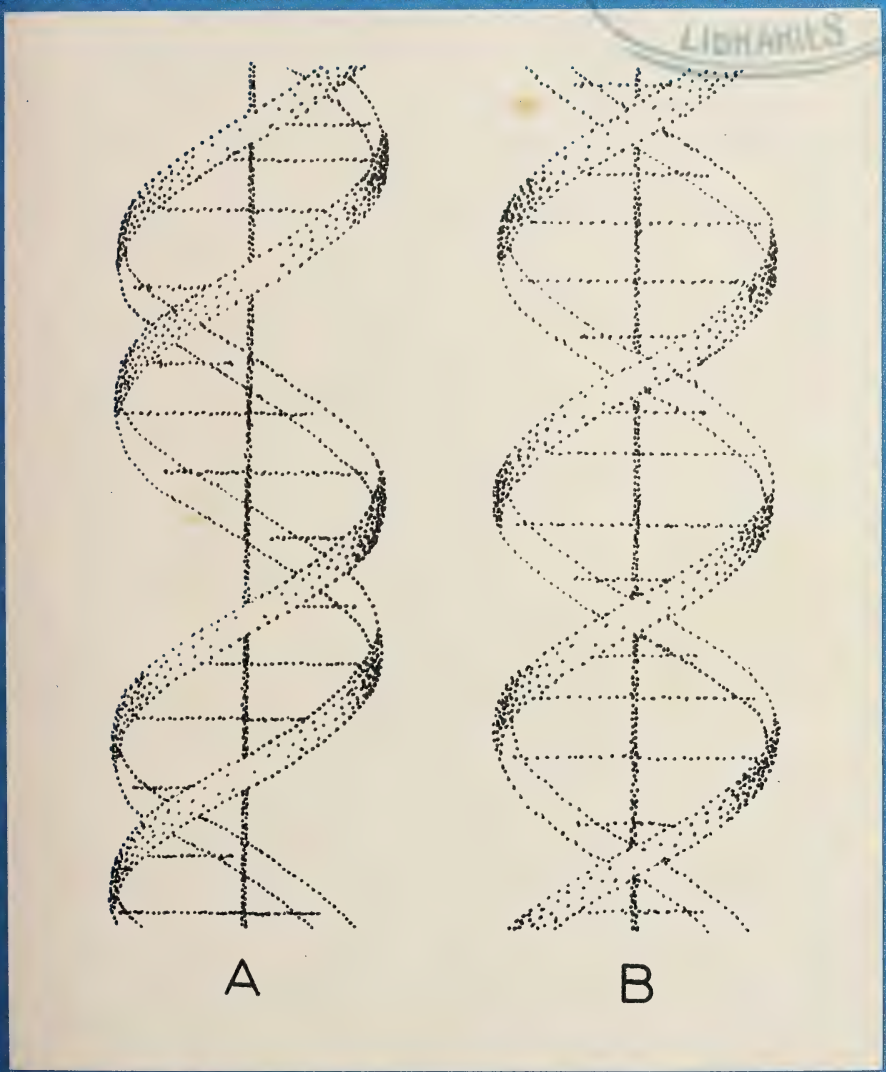

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NEW ENDOGENOUS RNA TUMOR VIRUSES

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ABSTRACT

Several new species of the family, *Phasianidae,* have been shown to possess endogenous RNA tumor virus helper activity by producing recombinants with the defective Bryan high-titer strain of Rous Sarcoma Virus (BH-RSV). The envelope properties of pseudotypes from cells of Chinese quail, Chukar, Mongolian Pheasant, and Swinhoe Pheasant differ from those previously encountered. Properties of the viruses, such as, plaque-forming ability in certain cell types, plating efficiency, host range, and interference patterns will be used to identify new viral subgroups. Endogenous RNA tumor viruses have been shown to arise spontaneously from normal embryo cultures in addition to their expression through complementation. Placing of the new pheasant viruses in a former or new subgroup of the subfamily, *Oncovirinae* is determined by immunological and biochemical studies.

INTRODUCTION

Many vertebrates that contain Oncovirus (Fenner, 1976), including several members of the avian family, *Phasianidae,* e.g. quail, partridge, domestic chicken, were grouped by antigenic similarities and host range. Pertinent avian species produce endogenous viruses spontaneously from normal cells (Vogt and Friis, 1971; Fujita, *et al.,* 1974; Crittenden, *et al.,* 1974) and through recombination (Hayward and Hanafusa, 1975; Hanafusa and Hanafusa, 1973) by the endogenous virus providing helper activity for defective exogenous virus. In this paper, newly discovered endogenous viruses, arising spontaneously and/or recovered after BH-RSV infection, from *Phasianidae* members will be described.

RESULTS

Helper Activity for the Defective Bryan High-Titer Strain of Rous Sarcoma Virus (RSV)

With chicken helper factor (CHF) positive and negative chick embryo fibroblast cultures serving as controls, RSV (-) was fused into embryo fibroblast cells

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with beta-propionolactone inactivated Sendai virus. Culture media, harvested every 2 to 3 days after the 7-day transformation period, were sonicated (to destroy whole cells) and used to perform focus assay infectivity tests. Infectious RSV pseudotypes were recovered from most RSV (-) transformed cells (Table 1) indicating that those cells produce viral glycoprotein which phenotypically mix with RSV (-) particles. Some cells even synthesize complete leukosis-like virus.

TABLE 1

Infectious RSV After Fusion of RSV (-) into Normal Cells

Cell Type and Abbreviation	Synthesis of Infectious Sarcoma Virus
Chinese quail (CHQ)	+
Green pheasant (GRP)	+
Silver pheasant (SIP)	+
Swinhoe pheasant (SWP)	+
Mongolian pheasant (MOP)	+
Ghighi pheasant (GIP)	+
Chukar (Stone partridge) (CHU)	+
Japanese quail (JAQ)	-
Peking duck (PED)	-
Moscovy duck (MOD)	-
Pigeon (PGN)	-
Goose (GSE)	-
Chicken	
C/O gs^{+}[1]	+
C/O gs^{-}[2]	-
C/B gs^{+}[3]	+
C/B gs^{-}[4]	-

[1] Represents chicken cell resistant to none of the avian sarcoma virus subgroups (i.e., susceptible to all ASV subgroups), group specific antigen positive.

[2] Represents chicken cell resistant to none of the avian sarcoma virus subgroups (i.e., susceptible to all ASV subgroups), group specific antigen positive.

[3] Represents chicken cell resistant to subgroup B avian sarcoma virus, group specific antigen positive.

[4] Represents chicken cell resistant to subgroup B avian sarcoma virus, group specific antigen negative.

Host Range of the New RSV Pseudotypes in Avian Cells

From comparison of focus-forming titers of RSV pseudotypes on embryo fibroblast cultures of certain broad range avian species, a differential host range was established (Table 2). Species which are closely related, taxonomically, have a similar RSV pseudotype host range. Similarities are seen with Green Pheasant (GRP) and Ringnecked Pheasant (RPV), which are of the same genus, as well

TABLE 2

Host Range of RSV Pseudotypes[1]

Cell type	Virus								
	R(RPV) envelope specificity F	R(GPV) envelope specificity G	R(CHQ)	R(GRP)	R(SIP)	R(SWP)	R(MOP)	R(GIP)	R(CHU)
Chicken									
C/O[2]	5.88	5.95	5.28	3.48	3.08	5.70	5.48	3.20	5.32
C/A[3]	5.62	5.92	5.18	4.00	2.18	2.78	1.54	2.20	1.60
C/BE[4]	5.45	5.96	5.18	3.85	2.86	5.48	5.56	3.08	5.26
C/C[5]	5.56	5.81	4.65	2.68	2.54	5.08	5.72	3.00	5.54
C/E[6]	5.86	6.00	5.68	2.75	3.38	5.95	5.85	3.30	5.86
Quail									
Chinese quail	3.70	5.26	<1.00	1.90	2.00	4.18	5.08	3.08	5.08
Japanese quail	5.93	5.89	4.68	2.20	2.68	4.78	4.98	3.89	4.95
Pheasant									
Golden pheasant	5.48	3.00	5.08	2.89	1.30	4.38	5.68	1.70	5.30
Ringnecked pheasant	4.86	5.68	5.26	1.90	1.60	4.32	5.32	2.70	5.32
Silver pheasant	5.32	5.95	5.56	2.65	2.00	4.26	5.18	2.86	4.78
Swinhoe pheasant	5.38	5.88	5.32	2.78	2.78	4.32	5.38	3.18	5.04
Mongolian pheasant	5.18	5.48	5.26	1.88	1.90	4.78	5.60	2.70	4.26
Ghighi pheasant	5.43	4.08	5.18	2.00	1.78	4.38	5.23	<1.00	5.20
Duck									
Peking duck	5.60	<1.00	5.15	3.48	<1.00	<1.00	<1.00	<1.00	<1.00
Moscovy duck	5.40	<1.00	5.69	3.40	<1.00	<1.00	<1.00	<1.00	<1.00
Goose	<1.00	<1.00	2.43	<1.00	<1.00	2.90	2.48	<1.00	2.70
Chukar	5.86	5.38	3.15	3.60	2.60	3.85	4.30	3.78	3.67

[1] Titer log focus-forming units/ml (FFU/ml). Resistance is indicated by parentheses.
[2] C/O as in Table 1.
[3] Chicken cell resistant to subgroup A, avian sarcoma virus.
[4] Chicken cell resistant to subgroup B and E, avian sarcoma virus.
[5] Chicken cell resistant to subgroup C, avian sarcoma virus.
[6] Chicken cell resistant to subgroup E, avian sarcoma virus.

as Ghighi (GIP) and Golden Pheasants (GPV) also of the same genus.The Silver Pheasant (SIP), although of a different genus, has the same host range as GPV and GIP. In contrast, the Mongolian Pheasant, (MOP), a member of the same genus as the Ringnecked Pheasant, (RPV) shows a dissimilarity by not infecting Peking duck. This factor is unlike RPV and GRP. Although the relationship is not clear, the Chukar (CHU) has almost the same host range as MOP. Swinhoe Pheasant (SWP) and Chinese Quail (CHQ) pseudotypes are unique, as is further substantiated below. Cellular resistance in homologous species is probably from endogenous viral interference, and, in heterologous species, it may be a genetic property of the cell.

Ability of New RSV Pseudotypes to Infect Mammalian Cells

New RSV pseudotypes were plated on 3 continuous mammalian cell lines (Table 3). All but 1 pseudotype failed to produce foci in the cultures. Chinese quail virus induced foci on *Microtus* and mouse cells with remarkably high efficiency; in this respect, R(CHQ), surpassed even the avian sarcoma virus SR-D, subgroup D, which was previously known to have the highest transforming efficiency in mammalian cells.

TABLE 3

Transforming Ability of RSV Pseudotypes for Mammalian Cells

Virus	Cell Type and Log FFU/ml			
	CEF[1]	MA[2]	3T3[3]	NRK[4]
R[5](CHQ)	5.48	3.60	3.08	<0.30
R(GRP)	4.48	<0.30	<0.30	<0.30
R(SIP)	3.08	<0.30	<0.30	<0.30
R(SWP)	5.70	<0.30	<0.30	<0.30
R(MOP)	5.59	<0.30	<0.30	<0.30
R(GIP)	2.86	<0.30	<0.30	<0.30
R(CHU)	5.48	<0.30	<0.30	<0.30
SR-D[6]	5.75	1.30	1.00	0.00

[1]Chick embryo fibroblasts.
[2]*Microtus agrestis* (European field vole) fibroblasts.
[3]3T3 cells clone A31 from Balb/c mice.
[4]Normal rat kidney fibroblasts.
[5]Rous sarcoma virus.
[6]Schmidt-Ruppin - subgroup D.

Polybrene Requirements for the New Pseudotypes

The polycation, Polybrene, shown to enhance absorption of all avian leukosis and sarcoma viruses, except subgroup A, onto chicken embryo fibroblasts (Toyoshima and Vogt, 1969), enhanced foci formation of cells infected with the new RSV pseudotypes (Table 4).

TABLE 4

Effect of Polybrene on Focus Formation

Virus	Cell	Virus Titer (Log FFU/ml)		Factor of enhancement[1]
		With Polybrene	Without Polybrene	
R(CHQ)	C/E	5.48	4.30	15.00
R(GRP)		4.48	3.18	20.00
R(SIP)		3.08	1.78	20.00
R(SWP)		5.70	4.40	20.00
R(MOP)		5.59	4.53	12.00
R(GIP)		2.86	2.00	07.00
R(CHU)		5.48	4.40	12.00
B77[2]		5.75	4.34	26.00
PR-A[3]		5.18	5.08	01.25

[1] Titer in the presence of 2μg of Polybrene in the growth medium at the time of infection over titer without Polybrene.

[2] Avian sarcoma virus strain B77.

[3] Prague strain – subgroup A.

Patterns of Interference Between Avian Leukosis Viruses of Different Subgroups and the New RSV Pseudotypes

Glycoproteins of previously classified avian leukosis viruses and the new RSV pseudotypes were compared by interference tests using (Type C/E or C/O) chick embryo fibroblasts (Table 5). Interference occurred with all new pseudotypes, except for R(SWP), R(MOP), R(CHU), and R(CHQ). These 4 new pseudotypes may be unrelated in envelope glycoprotein to known viruses or may be mixtures of different glycoprotein type viruses; they were, however, isolated from host cells taxonomically related to cells that produce viruses of previously established subgroups.

Isolation of Leukosis Virus from R(CHQ) and R(SWP)

A leukosis helper virus, capable of inducing specific homologous pseudotype interference, was recovered from both R(CHQ), and R(SWP) by the endpoint interference technique (Rubin and Vogt, 1962) (Table 6). R(CHQ) and R(MOP) may have contained less leukosis virus than sarcoma virus, and did not yield isolatable leukosis helper virus. In interference tests, viruses of subgroup A–G, did not interfere with CHQ or SWP virus (Table 7). Interference with their own pseudotypes suggested that CHQ and SWP viruses were not mixtures of viruses with different envelope types.

Spontaneous Release of Endogenous Viruses in Normal Avian Embryo Cultures

To reveal helper activity, supernatant from the normal cells listed in Table 2 was added to RSV(-) transformed cells of chicken, duck, and Japanese quail.

TABLE 5

Interference Patterns of RSV Pseudotypes

Cell Type	Interfering virus and envelope specificity	Challenge Virus													
		PR[7]-A	PR-B	PR-C	SR[8]-D	SR-E	R[9](RPV)	R(GPV)	R(GRP)	R(SIP)	R(GIP)	R(SWP)	R(MOP)	R(CHU)	R(CHQ)
C/E CEF[3]	None	120[1]	98	90	42	NT[2]	108	96	72	66	132	102	144	138	78
	RAV[4]-1(A)	0	90	100	20	NT	95	90	60	55	120	120	100	110	70
	RAV-6(B)	180	0	102	2	NT	90	180	105	72	144	210	200	200	120
	RAV-7(C)	132	123	0	30	NT	74	120	95	105	108	180	145	156	90
	CZAV[5](D)	120	0	60	0	NT	66	126	75	60	90	114	180	180	85
	RAV-61(F)	180	156	96	25	NT	0	180	0	45	70	120	240	120	15
	GPV[6]("G")	120	90	58	27	NT	120	0	47	0	0	150	180	120	75
C/O CEF	None	90	NT	NT	NT	87	NT	NT	150	180	96	110	72	128	90
	RAV-O(E)	96	NT	NT	NT	0	NT	NT	132	65	80	105	60	68	98
	RAV-60(E)	72	NT	NT	NT	0	NT	NT	90	78	150	60	120	150	72

[1] Foci/dish in a representative experiment. Interference is indicated by parentheses.
[2] Not tested.
[3] Chick Embryo Fibroblast.
[4] Rous Associated Virus (endogenous).
[5] Carr-Zilber Rous Associated Virus.
[6] Golden Pheasant Virus.
[7] Prague Strain.
[8] Schmidt-Ruppin.
[9] Rous Sarcoma Virus.

TABLE 6

Isolation of Helper Leukosis Viruses from R(CHQ) and R(SWP) Pseudotypes by Endpoint Interference

Dilution of Pseudotype	Foci formed by Challenge Virus		No Challenge
	RSV(CHQ)	RSV(SWP)	
RSV(CHQ)			
1:11,600	12	200	7
1:17,400	0	180	0
1:26,100	2	218	0
1:39,100	224	265	0
Uninfected	240	307	0
	PR-A	RSV(SWP)	
RSV(SWP)			
1:51,000	118	20	12
1:77,000	142	0	0
1:116,000	130	312	0
1:174,000	152	290	0
Uninfected	183	358	0

TABLE 7

Interference Patterns of New Endogenous Viruses

Cell Type	Interfering Virus and Envelope Specificity	Challenge Virus							
		PR-A[5]	PR-B	PR-C	SR-D[6]	R[7](RPV)	R(GPV)	R(CHQ[3])	R(SWP[4])
C/E	None	183[1]	150	240	180	208	288	240	307
	RAV[2]-1(A)	0	112	105	100	176	190	200	107
	RAV-2(B)	165	0	202	2	197	235	256	218
	CHQ virus	136	131	224	118	58	250	0	280
	SWP virus	60	180	200	54	132	300	180	0

[1] Foci/dish in a representative experiment. Interference is indicated by parenthesis.
[2] Rous associated virus.
[3] Chinese quail.
[4] Swinhoe pheasant.
[5] Prague strain - subgroup A.
[6] Schmidt-Ruppin - subgroup D.
[7] Rous sarcoma virus.

These cells, after 2 transfers, were assayed for infectious RSV pseudotype on the 7th day. Helper activity and hence spontaneously released endogenous virus was isolated from Chinese quail, Golden pheasant, and Ghighi pheasant, but not

from the others tested (Table 8). Spontaneously released Chinese quail virus and R(CHQ) pseudotypes showed the same interference pattern whereas, helper viruses from golden and Ghighi pheasant cells showed subgroup G interference.

TABLE 8

Spontaneous Release of Endogenous Virus in Cultures of Normal Avian Cells

Source of Culture Fluids	Synthesis of Infectious Virus on RSV(-) Cells (log FFU/ml)[1]		
	RSV(-)C/BE	RSV(-)PED[2]	RSV(-)Q[3]
Chinese quail	4.48	4.80	3.90
Golden pheasant	4.15	0.00	3.60
Ringnecked pheasant	0.00	0.00	0.00
Green pheasant	0.00	0.00	0.00
Silver pheasant	0.00	0.00	0.00
Swinhoe pheasant	0.00	0.00	0.00
Mongolian pheasant	0.00	0.00	0.00
Ghighi pheasant	2.00	0.00	1.30
Chukar (Stone partridge)	0.00	0.00	0.00
RAV-61 (control)[4]	4.70	4.34	4.30
None[5]	0.00	0.00	0.00

[1] Titrated on C/BE chicken cells.
[2] Peking duck.
[3] Quail
[4] Each RSV(-) line was infected with RAV-61.
[5] Supernatant of each RSV(-) line.

Plaque Formation

Plaques were produced by the pseudotypes from CHQ and SWP cells (Table 9); CPE for the former was first seen between 10 to 14 days and at 20 days in the

TABLE 9

Plaque Formation with New Endogenous Viruses

Virus	PFU/ml
CHQ virus, rescued	1.3×10^5
CHQ virus, spontaneous	<10
SWP virus, rescued	2.0×10^5
GPV, rescued	<10
GPV, spontaneous	<10
GIP virus, spontaneous	<10

latter. None of the spontaneously produced viruses formed plaques, suggesting that these viruses and their RSV pseudotypes are not genetically identical. Robinson (1976) observed a similar disparity with 2 subgroup E viruses, one of the viruses (RAV-60) being a recombinant with RAV-1.

DISCUSSION

Two groups of Avian Oncoviruses have been differentiated by lack of immunological cross reactivity and by lack of nucleic acid homology. Recently, a 3rd group or species (Hanafusa, *et al.,* 1976) has been added. The first 2 groups, avian leukosis-sarcoma viruses and reticuloendothelial viruses, unlike the 3rd group, do not show interaction by phenotypic mixing (Theilen, *et al.,* 1966; Maldonado and Bose 1971; Halpern, *et al.,* 1973; Kang and Temin, 1973; Moelling, *et al.,* 1975). The 3rd group was originally isolated from cells of the Pheasant genus, *Chrysolophus* (Hanafusa, *et al.,* 1976), and was designated subgroup G Fujita, *et al.,* 1974). Presently, a variant of the golden pheasant, Ghighi pheasant has been shown (by host range and interference tests) to have envelope specificity similar to previously discovered subgroup G viruses. Golden and Ghighi pheasants spontaneously produce endogenous viruses and are thereby a genetically pure source of new *Chrysolophus* pheasant viruses. Two of the 4 new helper viruses (CHQ, SWP), unrelated to previously described avian *Oncovirinae* in envelope properties, have been separated from their pseudotypes, but the other 2 (CHU and MOP) have not. The latter 2 may represent a mixture of known determinants as found with subgroup F and A envelope determinants in the Ringnecked pheasant (Temin and Kassner, 1976). It will be interesting to determine if other avian species harbor information for multiple glycoprotein types. Resistance of C/A, duck, and goose cells to R(CHU) and R(MOP) suggest subgroup A envelope character, but since there was no interference by subgroup A virus, a decision about their uniqueness or relationship to former subgroups will have to await isolation of the genetically homogeneous leukosis virus from the pseudotypes. Some structural proteins of CHQ virus differ from those of the avian leukosis, reticuloendotheliosis, and *Chrysolophus* virus. This fact, in conjunction with the recently discovered 3rd oncovirus group (Hanafusa, *et al.,* 1976) raises the possibility of the existence of other viral groups in the pheasant family. Detailed characterization of the new viruses is in progress utilizing radioimmunoassays and nucleic acid hybridization. The high plating efficiency of R(CHQ) in mammalian cells will provide an optimum test system for transformation mutants since in mammalian cells there is much less leakiness than in the avian system (Chen, *et al.,* 1977). *In vivo* studies in the natural avian host, as well as others, e.g. chicken, would be informative since all *Phasianidae* have been previously investigated only in culture.

CONCLUSIONS

Helper virus activity for RSV(–) was found in normal embryo fibroblast cultures of Ghighi, Green, Silver, Mongolian, and Swinhoe Pheasants and in Chinese quail and Chukar. The latter 4 species were found to specify unique envelope determinants. Helper viruses were isolated from Chinese quail and Swinhoe

pheasant pseudotypes. This study has uncovered an apparently new group of oncoviruses in the family *Phasianidae* and some of the viruses are produced spontaneously.

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THE *uvrC* GENE FUNCTION IS REQUIRED FOR EXCISION

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ABSTRACT

We have used isogenic strains of *uvrC*⁻ *E. coli* containing or lacking DNA polymerase I to study the role of the *uvrC* gene product in excision repair. The individual steps of incision, excision, repair synthesis and ligation in the excision repair process have been investigated using the toluene-treated cell system. The *uvrC*⁻ mutants execute incisions like wild-type cells, detected only in the presence of nicotinamide mononucleotide (NMN), a blocking agent for ligase activity. The *uvrC*⁻, *polA*⁺ cells do not exhibit the DNA polymerase I–mediated and NMN-stimulated repair synthesis observed in wild-type cells. In contrast, the *uvrC*⁻, *polA12* cells exhibit UV-specific, ATP-dependent synthesis like *polA12* strains. Excision of pyrimidine dimers is defective in *uvrC*⁻ mutants whether NMN is present or not in the reaction mixture. The return to normal molecular weight after incision accumulation is rapid in *uvrC*⁻, *polA*⁺ cells, like wild-type cells; the *uvrC*⁻, *polA12* cells demonstrate more reformation of high molecular weight DNA than *polA12* cells.

INTRODUCTION

Ultraviolet irradiation produces photoproducts which are potentially lethal to the cell. Among these, the pyrimidine dimers are the best understood photoproducts. A major pathway which results in the physical removal of pyrimidine dimers is the excision repair process. This repair process is dependent on *uvr* gene products as characterized by *E. coli uvr*⁻ mutants and may involve as few as 3 *uvr* gene products, *uvrA, uvrB,* and *uvrC*, for efficient removal of the damage. A possible mechanism for the excision repair process may involve the enzymatic events of incision, or the introduction of a phosphodiester break close to the dimer, excision of the dimers, reinsertion of nucleotides and finally covalent closure by ligase (Grossman, *et al.*, 1975).

The reported absence of incisions in *uvrA*⁻ and *uvrB*⁻ mutants suggests the involvement of *uvrA* and *uvrB* gene products in catalyzing incisions. The *uvrA,B* endonuclease has also been shown to be absent from *uvrA*⁻ and *uvrB*⁻ mutants (Braun and Grossman, 1974). However, no conclusive role of *uvrC* gene function has been determined, although the reported evidence suggests its involvement in

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incision (Taketo, *et al.*, 1972; Seeberg and Strike, 1976; Seeberg, *et al.*, 1976). In contrast, other *in vivo* studies show the formation of single-strand breaks in the DNA of *uvrC*$^-$ mutant after UV-irradiation (Kato, 1972) or mitomycin C (Otsuji and Murayama, 1972).

We have used the toluene-treated cell system to study the functional characteristics of *uvrC*$^-$ mutants. Toluene-treated cells are permeable to nucleotide substrates and appear to maintain the intactness of replicative and repair systems (Moses and Richardson, 1970; Bowersock and Moses, 1973). Recent studies from our laboratory and others have shown that toluene-treated cells can be successfully used for fractionating the individual steps of incision, excision, repair synthesis and reformation in excision repair.

METHODS

The detection of incisions or single-strand breaks and reformation of high molecular weight DNA were done by sedimentation of prelabeled DNA on alkaline sucrose gradients. The cells were labeled with [^{3}H]-thymidine (5 μCi/ml) and harvested during exponential growth. Toluene-treatment was done as described (Moses and Richardson, 1970). After toluene-treatment, UV-irradiation and incubation in reaction mixtures, the cells were directly lysed on top of 5 to 20% alkaline sucrose gradients. After centrifugation, the gradients were collected on paper strips, washed with cold trichloroacetic acid (10%), and the radioactivity in each fraction determined.

The excision of pyrimidine dimers was performed by using two-dimensional chromatography on pre-coated cellulose sheets. After TCA precipitation, the acid-insoluble material was hydrolyzed with 97% formic acid to convert the DNA nucleotides to bases. The data are expressed in terms of thymine dimers and total thymine in the DNA.

The levels of repair synthesis after UV-irradiation were monitored directly by acid precipitation, filtration and liquid scintillation counting (Bowersock and Moses, 1973).

RESULTS AND DISCUSSION

Seeberg and Strike (1976) have shown the lack of incisions in the DNA of UV-irradiated *uvrC*$^-$ cells using the sucrose-plasmolyzed *in vitro* cell system. In our studies, the experiments to look for incisions in *uvrC*$^-$, *polA*$^+$ and *uvrC*$^-$, *polA12* cells were done using toluene-treated cells. In toluene-treated cells, incisions can be demonstrated by omitting dNTPs or through the addition of NMN (Dorson and Moses, 1974; Waldstein, *et al.*, 1976; Dorson, *et al.*, 1978). Incisions can be monitored by the reduction in the size of the DNA on alkaline sucrose gradients. Under standard conditions for incision (no dNTPs), the *uvrC*$^-$, *polA*$^+$ cells did not show any appreciable reduction in the size of DNA. However, when

NMN was added to the reaction mixture, *uvrC*$^-$, *polA*$^+$ cells showed the same reduction in the size of the DNA as observed in wild-type (*polA*$^+$) cells. The *uvrC*$^-$, *polA12* mutants executed incisions only slightly less efficiently than the parental *polA12* strain even in the absence of NMN, and virtually the same reduction in the size of the DNA was observed in the presence of NMN. The *uvrC*$^-$ mutants showed the same kinetics of incision in the presence of NMN as observed in wild-type cells. The detection of incision in *uvrC*$^-$ mutants only in the presence of NMN suggests ligation of the breaks as a cause of failure to detect incisions. These results further indicate that the incision process is intact in *uvrC*$^-$ mutants.

A second approach was to study excision of pyrimidine dimers in *uvrC*$^-$ mutants. The excision of pyrimidine dimers in wild-type cells *in vitro* is dependent on ATP and the *uvrA,B* gene products (Deutsch, *et al.*, 1976). The excision of pyrimidine dimers is enhanced in the presence of simultaneously occurring repair synthesis (Dorson, *et al.*, 1978). We have studied excision of dimers in the presence of NMN in the reaction mixture; the addition of NMN would maximize the incision accumulation and repair synthesis. We failed to detect any excision of pyrimidine dimers in *uvrC*$^-$ (*polA*$^+$ or *polA12*) cells irrespective of the presence or absence of NMN (Table 1). These results indicate that *uvrC* gene function is required for excision of pyrimidine dimers and that the lack of incisions is not the basis for the failure of the excision of pyrimidine dimers.

TABLE 1

Dimer Excision in Toluene-Treated *uvrC*$^+$ and *uvrC*$^-$ Strains Exposed to 40 J/m^2 of UV-dose

Strains	Reaction Additions[1]	Incubation (min)	$\hat{XT}/T$[2] (%)	Dimers Present (%)
W3623 (*polA*$^+$)	ATP, dNTPs	0	0.057	100
		30	0.040	69
	ATP, dNTPs, NMN	0	0.041	100
		30	0.031	71
N17-7 (*uvrC*$^-$, *polA*$^+$)	ATP, dNTPs	0	0.034	100
		30	0.037	108
	ATP, dNTPs, NMN	0	0.051	100
		30	0.052	101
MM383 (*polA12*)	ATP, dNTPs	0	0.046	100
		30	0.031	69
RM76 (*uvrC*$^-$, *polA12*)	ATP, dNTPs	0	0.032	100
		30	0.033	103

[1] Complete reaction mixtures contain 66 mM KPO_4 (pH 7.4) buffer and 13 mM $MgCl_2$ in addition to 1.3 mM ATP, 33 μM dNTPs and 5 mM NMN (when needed).

[2] Dimer analysis was performed on TCA precipitable material. $\hat{XT}/T$ represents the ratio of radioactivity present in thymine dimers to radioactivity present in total thymine in the DNA.

The repair incorporation of nucleotides into DNA after UV-irradiation is approximately 30% of replication in *pol*A^- toluene-treated cells; this repair incorporation is much lower in cells containing DNA polymerase I (Moses and Moody, 1975). The low repair synthesis in *pol*A^+ cells has been related to the efficient interaction of polymerase I and ligase activities, resulting in short-patch repair synthesis (Dorson and Moses, 1978). The *uvrC*; *pol*A12 cells exhibited the same UV-induced, ATP-dependent repair synthesis observed in *pol*A12 cells, confirming the occurrence of incisions in *uvrC*$^-$, *pol*A12 cells. These results further suggest that the defect in *uvrC*$^-$, *pol*A12 cells does not inhibit repair synthesis. Recent studies from our laboratory have shown that NMN causes stimulation of repair incorporation after UV-irradiation in toluene-treated *pol*A^+ cells by inhibiting the ligation of breaks by polynucleotide ligase (Dorson and Moses, 1978). The results shown in Fig. 1 are typical for repair synthesis in toluene-treated W3623 (*pol*A^+) and N17-7 (*uvrC*$^-$, *pol*A^+) cells. The results presented in panel A show that UV-irradiation of the *pol*A^+ cells result in inhibition of replicative DNA synthesis. Stimulation of repair synthesis is demonstrable in the presence of NMN. The persistence of NMN-stimulated repair incorporation in the presence of N-ethylmalimide (NEM), an inhibitor of DNA polymerase II and III but not DNA polymerase I, suggests that the NMN-stimulated repair synthesis is mediated by DNA polymerase I. The addition of NEM to reaction mixtures containing NMN causes a large reduction in replicative DNA synthesis.

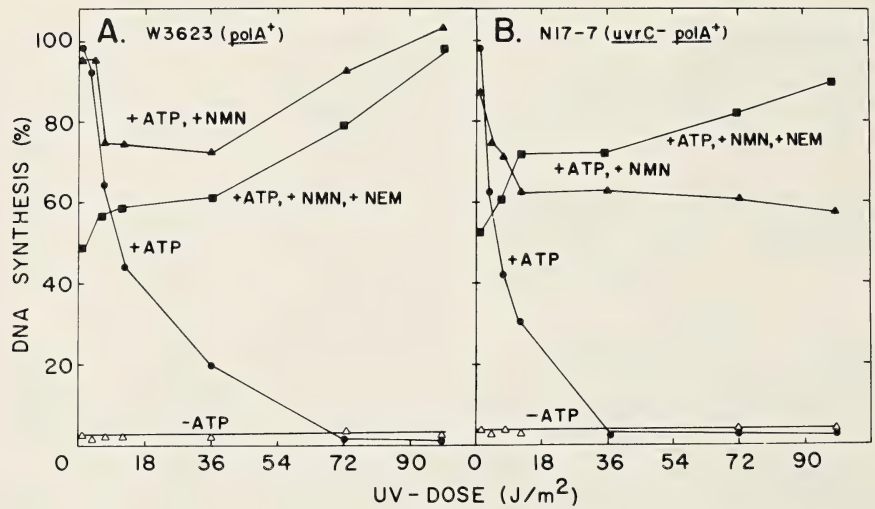

Figure 1. Comparison of NMN stimulation of repair synthesis in W3623 (*pol*A^+) and N17-7 (*uvrC*$^-$, *pol*A^+). Cells of strains W3623 and N17-7 were grown in L-broth to 1.0×10^9 of cells/ml, toluene treated at 1×10^{10} cells/ml cell density with 1% toluene, washed free of toluene and resuspended in 50 mM KPO_4 (pH 7.4) at 2.5×10^{10} cells/ml. The cells were irradiated by rapid shaking on a platform in coldroom at 0.8 J/m^2/sec with a lamp emitting at 254 nm. At various time intervals, portions were withdrawn and added to reaction mixtures (0.3 ml) containing 66 mM KPO_4 (pH 7.4), 13 mM $MgCl_2$, 1.3 mM ATP, and 33 μM dCTP, dGTP, dTTP and α-[^{32}P]dATP(90 cpm/pmole). NMN and NEM were present at 5 mM and 10 mM respectively, when required. All reactions were incubated for 30 min at 37 C, terminated with trichloroacetic acid and radioactivity determined.

Surprisingly, the NMN-induced stimulation of repair synthesis is not large in *uvrC*$^-$, *polA*$^+$ cells (panel B) compared with that in wild-type cells (panel A). The addition of NEM to reaction mixtures containing NMN does not also induce much stimulation of repair incorporation above the levels observed in the presence of only NMN.

The reformation of high molecular weight DNA in *uvrC*$^-$ mutants has been analyzed by sedimentation on alkaline sucrose gradients. We observed efficient restoration of high molecular weight of DNA in *uvrC*$^-$, *polA*$^+$ cells with better reformation of high molecular weight DNA in *uvrC*$^-$, *polA12* cells compared with *polA12* cells. These results suggest the persistence of the right configuration for ligation after incision.

The results presented in Table 1 and Fig. 1 suggest that the defect in excision of pyrimidine dimers might block the repair synthesis in *uvrC*$^-$, *polA*$^+$ cells by affecting nick-translation or strand-displacement. Nick translation requires addition of nucleotides by DNA polymerase I to the 3-hydroxyl termini with simultaneous release of nucleotides from 5' end terminus by 5' $\rightarrow$3' exonuclease (Kelly, *et al.*, 1969). The failure of excision of pyrimidine dimers in *uvrC*$^-$ mutants might block strand displacement, although DNA polymerase I has been shown to perform strand displacement by itself (Masamune and Richardson, 1971).

CONCLUSIONS

Contrary to published reports, we have demonstrated that the incision process is intact in *uvrC*$^-$ mutants. Excision of pyrimidine dimers is defective in *uvrC*$^-$ mutants *in vitro*. We propose that the event of re-sealing is responsible for the failure to detect incision. Our results on reformation of high molecular weight DNA also indicate that in the absence of dimer excision, ligase will seal the juxtaposed ends which will mask the occurrence of incisions. Our results further suggest that the *uvrC* gene function may be a pre-excision function, such as an unwinding protein or an exonuclease.

ACKNOWLEDGEMENTS

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COMMENTS

Nairn: In a *uvrA*$^+$ *uvrB*$^+$ *uvrC*$^-$ strain would you expect a similar response to mutagenic treatments as is observed in the conventional excision repair-deficient strains.

Sharma: I presume you are referring to *uvrA*$^-$ or *uvrB*$^-$ mutants as the conventional excision repair-deficient strains. Yes, *uvrC*$^-$ strains have been shown to manifest a similar response to mutagenic conditions. Our results clearly show the defect in excision of dimers irrespective of the occurrence of incisions in *uvrC*$^-$ mutants. Such mutants have also been shown to be defective in removing mitomycin C$^-$ and psoralen-induced damage. Thus, the presence of the damage will make these mutants sensitive to mutagenic treatments like *uvrA*$^-$ and *uvrB*$^-$ mutants, which are primarily defective in incision.

Nairn: If the same response were seen would you expect it to be abolished in a *uvrC*$^-$ *recA*$^-$ strain.

Sharma: We don't know.

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FURTHER STUDIES ON INDUCTION OF DNA SYNTHESIS IN KB CELLS BY A RHINOVIRUS

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ABSTRACT

Contact-inhibited cultures of KB cells reveal a slight transient stimulation in synthesis of DNA within 3 hrs of inoculation with rhinovirus type 14 (RV14) and thereafter the rates of synthesis of DNA in either RV14-inoculated or mock-cell fluid-inoculated cell cultures are similar. The newly synthesized DNA appears some 3–4 hrs before progeny virus appears in these cells. RV14 also stimulated DNA synthesis at a similar time period in L–132 and another KB cell line, but not in HeLa cells. Mitomycin C, hydroxyurea and actinomycin D prevented RV14–induced stimulation of DNA synthesis, whereas phleomycin, cordycepin and ethidium bromide did not. As replication of virus in the presence of these inhibitors could not be correlated with the capacity for stimulation of synthesis of DNA, the latter process probably does not directly contribute to replication of virus. Inoculation of KB cells with nononcogenic RNA-containing viruses also resulted in a transient stimulation in synthesis of DNA within 3 hrs of virus inoculation, further suggesting that virus stimulation of DNA synthesis is a general cell response and is not directed by the virus genome. The present data extend previously published data (Griffith, *et al.*, 1978), which showed that the newly synthesized DNA is rapidly transported to the cytoplasm and that the biophysical properties of cytoplasmic DNA from mock-cell fluid- and RV14-inoculated KB cells are different from nuclear DNA of these 2 cell cultures. The present study lends additional support to the hypothesis that an early KB cell response to RV14 infection is synthesis of a specific class of nuclear DNA which is rapidly transported to the cytoplasm and this nascent DNA does not measureably contribute to replication of RV14.

INTRODUCTION

Although cytoplasmic DNA has been isolated from several kinds of animal cells (Bach, 1962; Bach and Johnson, 1967; Radloff, *et al.*, 1967; Bond, *et al.*, 1969; Schneider and Kuff, 1969; Bell, 1969; Hall, *et al.*, 1971; Lerner, *et al.*,

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1971; Bell, *et al.*, 1972; Koch and von Pfeil, 1972; Smith and Vinograd, 1972; Meinke, *et al.*, 1973), the role of this DNA is unknown. As cellular DNA synthesis is not appreciably altered following infection of cells by picornaviruses until late in the growth cycle, it was of interest to find that rhinovirus type 14 stimulated DNA synthesis in contact-inhibited KB cells (Griffith, *et al.*, 1978). In the latter studies, we showed that RV14 induced a statistically significant transient stimulation in DNA synthesis within 3 hrs of inoculation and this DNA rapidly appeared in the cytoplasm. Infectious virus particles were required as the stimulus at the optimum temperature (34.5 C) for virus replication. Several biophysical criteria showed that the nascent cytoplasmic DNA species extracted from RV14- or mock-cell fluid-inoculated KB cells did not differ, but that cytoplasmic DNA from either type of cell culture could be differentiated from nuclear DNA extracted from these 2 cell cultures. In the present paper we present additional studies on the origin and properties of the cytoplasmic DNA extracted from KB cell cultures.

MATERIALS AND METHODS

Methods for the culture of all cell lines, for propagation and assay of RV14, and for infection of contact-inhibited cell cultures were previously described (Griffith, *et al.*, 1978). Propagation and plaque assays were described for coxsackievirus B3 (Trousdale, *et al.*, 1977), mengovirus (Gauntt and Lockart, 1966), and for reovirus type 3 and vesicular stomatitis virus (Gauntt, 1973a). As the procedure for detecting nascent DNA synthesis in RV14-inoculated cells is important in understanding the results, we will briefly outline the virus-infection and DNA assay procedure. Approximately 3 x 10^6 KB, HeLa, or L-132 cells in MEM (Eagle's minimal essential medium) containing 5% calf serum were seeded to 60 mm plastic tissue culture dishes. The cell cultures were then grown to a contact-inhibited state at 37 C for 24 to 48 hrs in a 5% humidified CO_2 incubator. Growth fluids were removed and the cell cultures were washed twice with VGM [virus growth medium: MEM containing 1% heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml)] and incubated for 24 hrs in VGM prior to inoculation with virus or mock-cell fluids. Cell viability, as determined by trypan blue exclusion, was 95% or greater in every experiment at the time of inoculation. Cell cultures were inoculated with 0.2 ml of virus [a minimum of 20 plaque-forming units (p.f.u.)] or mock-cell fluids. It is emphasized that fluids used in the mock preparations were prepared in an identical manner and at the same time as the virus-containing fluids that were used in each experiment. Duplicate cell cultures of both virus and mock-cell fluid-inoculated cultures were incubated with 1 μCi/ml of [^{3}H]-thymidine (20 Ci/mM) for 1 hr at various times post-inoculation (p.i.). At the end of the pulse period the cells were harvested by scraping into the radiolabelled medium and centrifuging the cells at 3000 x g for 5 min at 4 C. After washing the cells

3 to 5 times with 10 volumes of phosphate-buffered saline, each cell pellet was taken up in 1 ml of reticulocyte standard buffer (0.01 M NaCl, 1.5 mM $MgCl_2$, and 0.01 M Tris, pH 7.4) and 0.5% sodium dodecyl sulfate. An equal volume of 10% trichloroacetic acid (TCA) was added and after 10 min at 0 C, the TCA-insoluble precipitates were centrifuged at 3000 x g for 10 min at 0 C. Each sample was washed 5 times with cold 5% TCA, the pellets were disrupted with the aid of a vibration stirrer, the precipitates were solubilized by addition of 0.5 ml of NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) and radioactivity was measured on 100 μl of sample. [^{3}H]-thymidine counts in TCA-insoluble material in each sample were normalized by 1 of 2 methods as follows. Cells were grown to a contact-inhibited state and then cultured an additional 24 to 48 hrs in maintenance medium containing 0.01 μCi/ml of [^{14}C]-uridine without serum. These cell cultures were washed twice with phosphate-buffered saline and then inoculated with virus or mock-cell fluids. The TCA-insoluble [^{14}C] counts in each sample were taken as an indication of the amount of nucleic acids actually processed/sample and [^{3}H] in all samples was then corrected relative to the content of [^{14}C]. In another method, TCA-insoluble counts in [^{3}H]-thymidine were normalized to duplicate cell counts of each individual sample immediately following cell culture harvest at the end of the pulse period.

RESULTS AND DISCUSSION

The time course of replication of RV14 in contact-inhibited KB cell cultures was initially examined. Replication proceeded in these KB cell cultures with a similar growth curve (Table 1) as previously reported for low density KB cell cultures (Gauntt, 1973b; Gauntt, *et al.*, 1973). Low density refers to 2 to 3 x 10^6 cells/60 mm petri dish, compared to 4 to 6 x 10^6 cells/60 mm petri dish for contact-inhibited cell cultures. Allowing the cells to become contact-inhibited did not alter the capacity of these cells for replication of RV14. Note that progeny virus particles were not detected in infected cells until after the 5th hr of infection. The effect of RV14 on DNA synthesis in these cell cultures provided a surprising but reproducible result. The results of these experiments are shown in Fig. 1. The rates of incorporation of [^{3}H]-thymidine into TCA-insoluble material in RV14-inoculated cell cultures, compared with cell cultures inoculated with mock-cell fluids, showed a 3.4-fold increase in DNA synthesis during the initial period at 1 to 2 h p.i. Since curves of DNA synthesis in RV14- and mock-cell fluid-inoculated cultures showed similar rates of accumulation of [^{3}H]-thymidine into DNA after the initial increase in DNA synthesis had taken place, the data show that a further increase in DNA synthesis in RV14-inoculated cell cultures above that observed in mock-cell fluid-inoculated cell cultures did not occur. Turnover in the newly synthesized DNA, if it occurred, was at the

TABLE 1

Effect of Metabolic Inhibitors on Yields of RV14 and on Induction of DNA Synthesis by RV14 in KB Cell Cultures.[1]

Inhibitor (μg/ml)	Virus Yield[2] (p.f.u./cell)	Control %	RV14-INDUCED DNA SYNTHESIS[3] cpm virus-inoculated / cpm mock-cell fluids			DNA SYNTHESIS IN UNINF. CELLS cpm mock-challenged[4,5] / cpm mock-challenged[6]		
			0.1 h p.i.	1-2 h p.i.	2-3 h p.i.	0.1 h p.i.	1-2 h p.i.	2-3 h p.i.
Control	246	100.0	1.80	2.20	2.00	1.00	1.00	1.00
Phleomycin (20)	233	94.7	1.11	2.00	1.00	1.50	1.00	1.63
Mitomycin C (14)	146	59.3	0.92	0.50	0.85	0.33	0.95	0.63
Ethidium Bromide (1)	111	45.1	1.16	7.00	0.70	1.00	0.60	0.90
Hydroxyurea (10^{-3} M)	110	44.7	1.00	1.16	1.00	0.33	1.05	1.36
Cordycepin (25)	97	39.4	1.00	3.00	0.72	1.33	1.00	2.28
Actinomycin D (5)	35	14.2	1.53	1.56	0.66	0.21	1.00	0.55

[1] Cell cultures were treated with the metabolic inhibitor for 2 h p.i.
[2] Virus yields were measured in cell cultures harvested at 18 h p.i.
[3] cpm in [^{3}H]-thymidine incorporated into TCA-precipitable DNA/sample were corrected to the number of cells/sample.
[4] cpm levels in mock-cell samples ranged from 470 to 18,442 cpm.
[5] Mock-infected cells treated with inhibitor.
[6] Mock-infected cells not treated with inhibitor.

same rate in both cultures. Previous experiments (Griffith, *et al.*, 1978) established that the phenomenon of an increase in synthesis of DNA in RV14-inoculated KB cell cultures above control cell cultures occurred maximally between 1 and 2 h p.i. in approximately 90% of 52 separate experiments. Statistical analyses of these results established that ratios (RV14-inoculated/mock-cell fluid-inoculated KB cells) of 1.61 or 1.54 using normalization of [^{3}H]-thymidine counts in DNA to cell number or to [^{14}C]-uridine in RNA, respectively, are significant at the 95% confidence level. Ratios of statistical significance are underlined in succeeding tables. The virus-induced event(s) leading to increased DNA synthesis occurs some 2 to 3 hrs prior to the times of detection of viral RNA polymerase activity (5 h p.i.) (Griffith and Gauntt, 1975), and synthesis of viral RNA (5 h p.i.) (Gauntt, 1973b) in infected KB cell cultures. The induction of cell DNA synthesis by RV14 is therefore the earliest measurable effect of virus infection on KB cell macromolecular synthesis. The event(s) leading to induction of DNA synthesis occurs during the period of absorption and uncoating of infectious particles. In the RV14-KB cell culture system, adsorption of particles is linear for up to 2 hrs after challenge and approximately half of all infectious particles are attached by 1 h p.i., and more than 90% of infectious virus in a challenge inocula containing 10 p.f.u./cell are attached by 2 h p.i. (Gauntt, *et al.*,

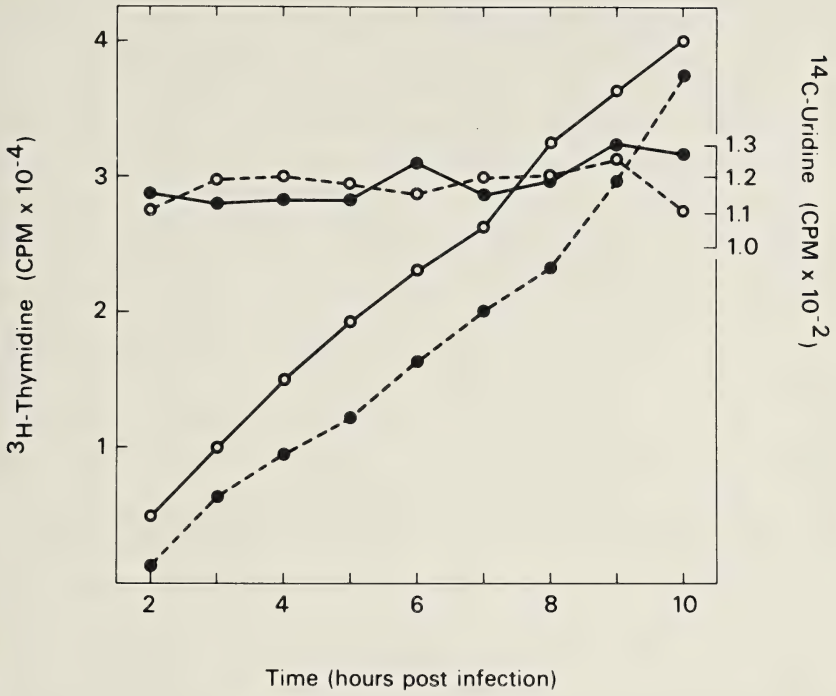

Figure 1. DNA synthesis in KB cell cultures inoculated with RV14 or mock-cell fluids. Each point represents the cumulative total of acid-insoluble [^{3}H] counts in DNA synthesized up to that time. [^{3}H] thymidine in DNA from cells inoculated with mock-cell fluids (●--●) or RV14 (0–0). Efficiency of extraction of DNA from each sample was monitored by recovery of TCA-insoluble [^{14}C]-uridine-labeled RNA from cells incubated in presence of [^{14}C]-uridine for 24 hrs prior to 0 time in each sample. [^{14}C]-counts in RNA from cells inoculated with mock-cell fluids (●–●) or RV14 (0--0).

1973). Uncoating of RV14 particles rapidly occurs following attachment to HeLa cells (Lonberg-Holm and Korant, 1972) and to KB cells (Gauntt, *et al.*, 1973), suggesting that active viral genomes are immediately available for translation, transcription, and influencing overall metabolic event(s).

The stimulation of incorporation of [^{3}H]-thymidine into TCA-insoluble DNA in contact-inhibited HeLa, L-132 and 2 lines of KB cells following inoculation with 50 p.f.u./cell of RV14 was measured. A statistically significant increase in DNA synthesis was found in both KB cell lines and in L-132 cells, but not in HeLa cells. Thus, stimulation of DNA synthesis by RV14 is not limited to a single cell line nor 1 cell strain.

Several experiments involving the use of several metabolic inhibitors were next performed in an attempt to provide information on whether induction of

DNA synthesis was required for production of infectious virus. Quantitative measurements of the effect of phleomycin, mitomycin C, cordycepin, hydroxyurea, ethidium bromide, and actinomycin D on RV14-induced DNA synthesis and virus yields were made as follows. KB cells were treated for 2 hrs with the inhibitors prior to challenge with RV14 or mock-cell fluids. Inhibitors were present at the indicated concentrations in the cell cultures throughout the experiments. Uninfected cell cultures were 99% viable 24 hrs after incubation with each inhibitor, as assessed by the trypan blue dye-exclusion test. Treated and untreated contact-inhibited KB cells were inoculated with 50 p.f.u./cell of RV14 or mock-cell fluids in the presence or absence (control) of the inhibitors. Incorporation of [^{3}H]-thymidine into TCA-insoluble material of both virus and the mock-fluid inoculated culture cells was measured as previously described and the results are presented in Table 1. DNA synthesis in uninfected cells was inhibited by mitomycin C and ethidium bromide as expected, whereas hydroxyurea and actinomycin D showed an initial inhibition of DNA synthesis, a result similarly reported for actinomycin D in HeLa cells (Clarke and Ellen, 1966). Phleomycin and cordycepin enhanced DNA synthesis in uninfected cells at 2 to 3 hrs; however, this time is later than the observed RV14-induced stimulation of DNA synthesis. Phleomycin, a water-soluble antibiotic that inhibits replication of mengovirus in Novikoff rat hepatoma cells at the concentrations used in these studies (Korbecki, 1973) surprisingly did not effect replication of RV14 or the RV14-induced stimulation of DNA synthesis. Differences in uptake or metabolism of this antibiotic by the 2 different cell types may account for the differential effect of phleomycin on replication of these 2 picornaviruses, rather than subtle differences in their replication patterns. Both mitomycin C and hydroxyurea prevented the RV14-induced stimulation of DNA synthesis and only slightly reduced yields of virus. In contrast, cordycepin and ethidium bromide only slightly reduced virus yields, but did not interfere with the RV14-induced stimulation of DNA synthesis. Actinomycin D did not permit a statistically significant level of stimulation of DNA synthesis by RV14 and appreciably reduced replication of the virus, the latter result in agreement with previous data (Gauntt, 1977). Taken as a whole, these results suggest that there is no direct correlation between the transient stimulation in DNA synthesis induced by RV14 and the replication of RV14, suggesting that the virus-induced event that leads to enhanced DNA synthesis is not critical to the sequence of events in RV14 replication.

KB cell cultures were challenged with several different viruses to determine whether other RNA-containing viruses could induce DNA synthesis. Contact-inhibited KB cell cultures were challenged with 50 p.f.u./cell of RV14, mengovirus, coxsackievirus B3, reovirus type 3 and vesicular stomatitis virus (VSV). The capacity of these viruses for stimulating DNA synthesis is shown in a typical experiment presented in Table 2. RV14, coxsackievirus B3 and reovirus type 3 induced DNA synthesis, whereas VSV did not. Note that VSV replicated very poorly in these KB cells. These results show that picornaviruses (mengovirus

and coxsackievirus B3) other than RV14 and a virus with a different scheme of replication (reovirus type 3) can induce DNA synthesis in KB cells.

TABLE 2

Stimulation of DNA Synthesis in KB Cell Cultures Challenged with Several Different Viruses

Virus	Virus-Induced DNA Synthesis (cpm virus-inoculated)[1] / (cpm mock-cell fluids)[2] 0-1 h p.i.	1-2 h p.i.	2-3 h p.i.	Virus Yield (p.f.u./cell)
RV-14	1.75	2.00	0.88	107
mengovirus	0.79	2.00	1.00	200
VSV	0.75	1.00	1.21	13
coxsackievirus B3	0.63	1.56	1.33	2,000
reovirus type 3	1.33	1.65	1.15	57

[1] cpm in ^{3}H-thymidine incorporated into TCA-precipitable DNA/sample were corrected to the number of cells/sample.

[2] cpm levels in the mock-cell fluid inoculated samples ranged from 523 to 9,392.

We have previously shown (Griffith, *et al.*, 1978) that the newly synthesized DNA is quickly transported to the cytoplasm, based on cell fractionation and electron microscope radioautographic methods. The cytoplasmic DNA from either mock-cell fluid- or RV14-inoculated KB cells could be differentiated from nuclear DNA extracted from these same 2 types of cell cultures by differences in buoyant densities in CsCl and CsCl-ethidium bromide gradients (Griffith, *et al.*, 1978). Furthermore, cytoplasmic DNA from either cell culture could be differentiated from nuclear DNA of these same cell cultures by DNA-DNA reassociation studies (Griffith, *et al.*, 1978). These latter results are summarized in Table 3. The $Cot_{1/2}$ value of cytoplasmic DNA was about 10-fold less complex than the $Cot_{1/2}$ value for nuclear DNA, thus ruling out random breakage of nuclei and leakage of DNA into the cytoplasm during the extraction procedure. These results suggest that inoculation of cells by RV14 does not result in induction of synthesis of new species of cell DNA, rather, RV14 in some manner stimulates an increase in synthesis of existing species of DNA which are rapidly transported to the cytoplasm. The $Cot_{1/2}$ value of the cytoplasmic DNA extracted from KB cells inoculated with RV14 or mock-cell fluids is quite different from the $Cot_{1/2}$ values expected for either mycoplasma (1.5), or SV40 DNA (2.6×10^{-3} to 1×10^{-2}) and is in agreement with the $Cot_{1/2}$ of 52 reported for the slowly reassociating fraction of a cytoplasmic species of DNA extracted from a diploid human lymphocyte cell line (Meinke, *et al.*, 1973). Reference to the plot of Britten and Kohn (1968) on genome size relative to $Cot_{1/2}$ of the DNA extracted from KB cells inoculated with either RV14 or mock-cell fluids corresponds to

TABLE 3

Comparison of $Cot_{1/2}$ from Different Sources of DNA Determined by Reference to Plot of DNA-DNA Reassociation of Britten and Kohn (1968).

Origin of DNA	Molecular Wt. (D)	$Cot_{1/2}$ [1]	Reference
Mitochondrial (average human)	1.0×10^7	4.5×10^{-2}	Helinski and Clewell (1971) Borst and Kroon (1969)
Mitochondrial (guinea pig)	1.1×10^7	5.0×10^{-2}	Hollenberg, *et al.*, (1970)
rat ascites tumor	2.5×10^{12}	8.0×10^3	Wetmur and Davidson (1968)
SV40 virus	3.3×10^6 to 4.2×10^6	1.0×10^{-2} to 2.6×10^{-3}	Wetmur and Davidson (1968) Gelb, *et al.*, (1971)
human leukocyte	-	7.6×10^3	Saunders, *et al.*, (1972)
human lymphocyte diploid cell line	-	52	Meinke, *et al.*, (1973)
mycoplasma	-	1.5	Bak, *et al.*, (1969)
KB cytoplasmic[2]	-	35-100	Griffith, *et al.*, (1978)
KB nuclear[2]	-	800-1000	Griffith, *et al.*, (1978)

[1] $Cot_{1/2}$ are approximate values calculated from reference plot by Britten and Kohn (1968) or calculated from data of Griffith, *et al.*, (1978).

[2] No distinction could be made between DNA extracted from cells inoculated with RV14 or mock-cell fluids.

a complexity of about 10^7 nucleotides. Therefore, the cytoplasmic DNA from KB cell cultures inoculated with RV14 or mock-cell fluids is over 12-fold more complex than a typical mycoplasma genome which is composed of about 8×10^5 nucleotide pairs (Bak, *et al.*, 1969). Similar arguments make unlikely viral contamination as the origin for cytoplasmic DNA in KB cells. The theoretical $Cot_{1/2}$ value for a non-repetitive DNA, e.g., virus DNA with a molecular weight of 4.2×10^6 Daltons, is 2.6×10^{-3} (Gelb, *et al.*, 1971). Therefore, the cytoplasmic DNA from infected and mock-infected KB cells reassociates 20,000 times more slowly than would be expected for virus DNA of a similar size. The $Cot_{1/2}$ for the slow reassociating fraction of nuclear DNA was approximately 10^3, which is similar to that observed for unique-sequence DNA extracted from human lymphocyte cultures (Meinke, *et al.*, 1973) and human leukocytes (Sanders, *et al.*, 1972).

CONCLUSION

We have shown that inoculation of several cell lines with RV14 results in stimulation of synthesis of DNA. Studies with various inhibitors show no correlation between virus yields and stimulation in DNA synthesis, thus RV14-induced stimulation of DNA synthesis and rapid transport of the DNA to the cytoplasm (Griffith, *et al.*, 1978) does not appear to be required for replication of

this virus. Induction of synthesis of DNA by RV14 through an unknown mechanism(s) represents an addition to a long list of materials which stimulate DNA synthesis in cells, including: conditioned media (Austin, *et al.*, 1972), serum (Temin, *et al.*, 1972), insulin (Temin, 1967), nerve growth factor (Greene, *et al.*, 1968), and epidermal growth factor (Attardi, *et al.*, 1967). Induction of DNA synthesis by these latter factors occurs some 5-8 hrs after addition, in contrast to stimulation of DNA synthesis by RV14 which occurred generally within 2 hrs after inoculation of virus. A definitive role for these DNA molecules in cell survival, or perhaps perpetuation of virus genomes in some cells, awaits additional studies.

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COMMENTS

Nagyvary: One of the earliest events observed following infection by RNA viruses is a change in cyclic AMP levels in the cell. Could the stimulation of DNA synthesis observed early in RV14 infection of KB cells be related in some way to levels of cyclic AMP in the cells?

Gauntt: It is known that cyclic AMP levels fall rapidly in cells following addition of agents (serum, insulin) that stimulate quiescent cells to synthesize DNA. The answer to your question is yes, the RV14-induced stimulation of DNA synthesis could be related to an alteration in cyclic AMP levels. HeLa cells in the G_2 phase (contact-inhibited) are known to have a minimal level of cyclic AMP.

R. Scott: Have you isolated DNA from the cell, post-infection, and determined to what extent it will associate with purified RNA from the virion?

Gauntt: We have not attempted hybridization between virion RNA and either cytoplasmic or nuclear DNA.

Henderson: Might the stimulation of DNA synthesis early in infection be a general response by the *cell* to infection, rather than DNA synthesis directed by the virus *per se*?

Gauntt: Yes, we believe that the DNA we are extracting from the cytoplasm is a cell response to virus infection. Several viruses other than RV14 stimulate a transient synthesis of DNA within 3 h p.i. and, as pointed out in the discussion, many other substances can induce DNA synthesis in cells. Cytoplasmic DNA from mock-cell fluid- or RV14-inoculated KB cells cannot be differentiated, but cytoplasmic DNA can be differentiated from nuclear DNA by several techniques, suggesting that only a specific class of nuclear DNA is transported

Gauntt: (Continued)
to the cytoplasm following RV14 infection. In addition, the experiments with the antibiotics suggest that replication of RV14 can proceed in either the presence or absence of induction of DNA synthesis by RV14.

Fralick: Have you tried competition hybridization as a means of comparison of cytoplasmic DNA isolated from infected cells with DNA from non-infected cells?

Gauntt: No, competition hybridization experiments between cytoplasmic DNA from RV14-inoculated and mock-cell fluid-inoculated cells were not conducted.

Garrard: Just by way of comment, a mixture of mitochondrial DNA and trace amounts of nuclear DNA, such as might arise from leakage, if analyzed for reassociation kinetics, would result in $Cot_{1/2}$ values similar to those you have presented. An homogeneous sample would be expected to follow 2 logs on a $Cot_{1/2}$ curve. Can you comment on the role of mitochondrial DNA in the reassociation experiments?

Gauntt: No radiolabelling of mitochondrial DNA was detected by electron microscopy autoradiography during our 1 hr ^{3}H-thymidine pulse experiments. No radiolabelled mitochondrial DNA species were detected following isopycnic centrifugation into CsCl or CsCl-ethidium bromide gradients. Thus, radiolabelled mitochondrial DNA is in minor concentrations and we think does not contribute to the $Cot_{1/2}$ values we reported for cytoplasmic DNA. Also, the $Cot_{1/2}$ values reported in the literature for mitochondrial DNA are quite different (see Table 3) from anything we measured.

Plunkett: One way to account for the apparent increase in DNA synthesis early in infection is that the infected cells have a higher specific activity of endogenous dTTP. This could result, for example, by suppression of host dTTP synthesis viral infection. Have you attempted to compare the infected and noninfected cells by measuring dTTP specific activities in each case?

Gauntt: We have not measured dTTP specific activities in any type of cell, however, ^{3}H-thymidine pool sizes in the nucleus or in the cytoplasm did not appear to be altered following infection with RV14.

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THE CATALYTIC MECHANISM OF METHYLGLYOXAL SYNTHASE

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ABSTRACT

Methylglyoxal synthase from *P. vulgaris* was isolated to homogeneity and utilized to assess the reaction mechanism. [^{14}C] dihydroxyacetone phosphate labeled in either the C-1 or C-3 position was enzymatically synthesized, isolated and utilized as a substrate for the enzyme. After reaction with the enzyme, the methyl carbon of methylglyoxal was identified as CHI_3 by the iodoform reaction. The labeling pattern revealed that C-1 is dephosphorylated and reduced to the methyl group, while C-3 is oxidized to the aldehyde. Methylglyoxal was found to be noncompetitive with respect to dihydroxyacetone phosphate, while inorganic phosphate was competitive and transformed the dihydroxyacetone phosphate saturation kinetics from hyperbolic to sigmoidal. The enzyme was inactivated by freezing, and phosphate stabilized the enzyme toward both cold- and heat-induced denaturation. The phosphate moiety of the substrate appears to be required for binding, since the synthase is competitively inhibited by a variety of phosphorylated compounds, but not by their nonphosphorylated counterparts. 3-Bromo- and 3-iodoacetol phosphates irreversibly inactivate methylglyoxal synthase. The substrate, dihydroxyacetone phosphate, and inorganic phosphate protect against the inactivation. Reduction of the enzyme-inactivator complex with [^{3}H]-$NaBH_4$, followed by amino acid analysis, suggested that the modification occurred through an acid-labile linkage, and the modified residue(s) was either glutamate or aspartate. No metal ion or cofactor was found to be involved in the enzyme-catalyzed reaction. Based on these observations, a mechanism is proposed in which the enzyme first catalyzes the ketoenol tautomerization to the hydrogen-bonded enol which facilitates the internal oxidation-reduction and phosphoester cleavage with C-O bond breaking, followed by an ordered sequential release of products with methylglyoxal being the first product.

INTRODUCTION

The biological significance of methylglyoxal has been a puzzle since the early part of the century. It has been observed in normal and abnormal tissues and cells,

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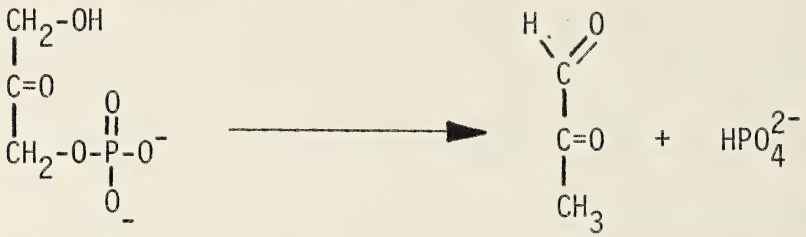
and proposed to be involved in the regulation of cell growth. Although the glyoxalase system has been proposed as a pathway for the catabolism of this highly toxic alpha-ketoaldehyde, the method(s) by which this compound is formed *in vivo* has remained unclear. Thus, when Cooper and Anderson (1970) described a methylglyoxal synthase (EC 4.2.99.11) which catalyzed the reaction



Dihydroxyacetone phosphate Methylglyoxal

it was of considerable interest. This enzyme, along with glyoxalase I and II, and D-lactate oxidase, provided a potential glycolytic bypass from dihydroxyacetone phosphate to pyruvate.

The first enzyme of this glycolytic shunt, methylglyoxal synthase, appears to be the rate-limiting reaction and has been suggested to be allosterically regulated by levels of inorganic phosphate (Cooper, 1974). Moreover, the reaction catalyzed by this enzyme appears to be rather unique in catalyzing both an internal oxidation-reduction and phosphorolysis. In order to better understand this critical enzyme and its biological function, we have sought to gain insight into its mechanism of catalysis.

MATERIALS AND METHODS

Methylglyoxal synthase was purified from *Proteus vulgaris* by a combination of DEAE cellulose ion exchange chromatography, gel filtration and isoelectric focusing as described earlier (Tsai and Gracy, 1976). The enzyme was crystallized and shown to be homogeneous by gel electrophoresis in the presence and absence of sodium dodecyl sulfate and by analytical ultracentrifugation.

RESULTS AND DISCUSSION

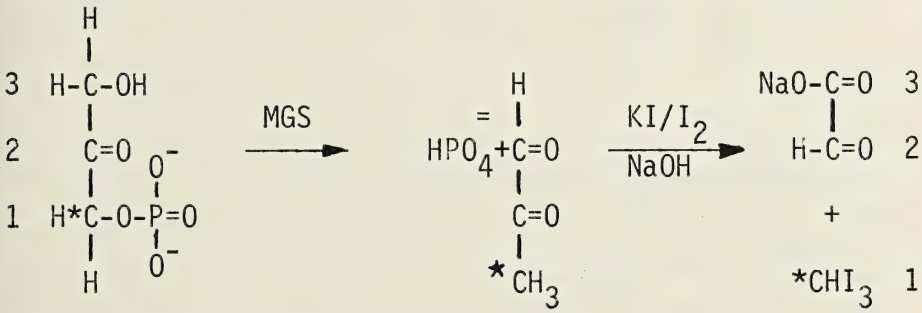
The substrate specificity of the enzyme was examined in detail. The enzyme does not catalyze the formation of methylglyoxal from glyceraldehyde 3-phosphate, dihydroxyacetone or glyceraldehyde. A variety of phosphorylated glycolytic intermediates are powerful competitive inhibitors of the enzyme, but the nonphosphorylated analogues are neither substrates nor competitive inhibitors. Thus, it appears that a phosphate-binding group must be located in the catalytic

center. Exhaustive studies have shown that the enzyme does not contain or require either metal ions or any coenzyme.

Next, we asked the question, which carbon is oxidized and which carbon is reduced? There was no way of knowing which carbon, C-1 (with the PO_4 group) or C-3 (with the free hydroxyl) is reduced to the methyl group and which carbon is oxidized to the aldehyde. Radioactive dihydroxyacetone phosphate labeled in the C-1 position was enzymatically synthesized from [6-^{14}C] glucose, while C-3 [^{14}C]-dihydroxyacetone phosphate was formed from D-[3,4-^{14}C] glucose (Yuan and Gracy, 1977). In 2 parallel experiments we incubated these 2 substrates with the enzyme. Both experiments contained identical amounts of radioactively labeled substrates of identical specific radioactivity and equal amounts of enzyme. The reactions were stopped, and the iodoform reaction carried out by treating with iodine in alkali. Under these conditions, the methyl group of methylglyoxal was precipitated as iodoform. The precipitates were collected, washed and resolubilized and counted by liquid scintillation.

When C-3 labeled dihydroxyacetone phosphate was the substrate, essentially no radioactivity was found in the iodoform precipitate. On the other hand, when C-1 was labeled, the resulting methyl group of methylglyoxal was radioactive as indicated by the radioactive iodoform.

Thus, it was clear that it is the phosphorylated carbon of dihydroxyacetone phosphate which is reduced to the methyl group of methylglyoxal, while the hydroxylated carbon is oxidized to the aldehyde, as indicated below.



Since there are 2 products in the methylglyoxal synthase-catalyzed reaction, we wanted to know whether both products are released from the enzyme randomly or if there is an ordered release, and if so, which product is released first. Product inhibition studies were utilized to answer this question. Product inhibition by inorganic phosphate was nonlinear, since it is an allosteric effector (Yuan, *et al.*, 1976, 1977). However, the nonlinear inhibition is overcome by dihydroxyacetone phosphate in a competitive fashion, indicating that dihydroxyacetone phosphate and inorganic phosphate both bind to the free enzyme. On the other hand, inhibition by methylglyoxal was noncompetitive. Since only the first substrate and last product should be mutually competitive, these results suggested an ordered release of products with methylglyoxal being released first, followed by the release of inorganic phosphate.

These data were further corroborated by the observation that inorganic phosphate in the absence of methylglyoxal markedly stabilized the enzyme against denaturation, suggesting that a E-Pi complex can form, even in the absence of methylglyoxal. Methylglyoxal, on the other hand, had no effect on the stability of the enzyme.

Next, we sought to gain additional information regarding the active center of the enzyme and explored the use of highly reactive substrate analogs of dihydroxyacetone phosphate, the haloacetol phosphates. We had reasoned that 1 of the analogs might bind in the active center and then react with a reactive nucleophile in the catalytic center to form a covalent affinity label. Chloroacetol phosphate did not inactivate the enzyme, but was found to be a strictly competitive inhibitor, suggesting that it binds in the active center. Both bromo- and iodoacetol phosphates irreversibly inactivate the enzyme in a pseudo first-order fashion. Figure 1 shows a semi-log plot of the loss of enzyme activity with time at various concentrations of iodoacetol phosphate. The inactivation by bromo- or iodoacetol was prevented by the presence of the substrates Pi or dihydroxyacetone phosphate.

There are 2 possible kinetic schemes of inactivation.

$$\mathrm{XAP} + \mathrm{MGS} \xrightarrow{k_{2nd}} \mathrm{XAP\text{-}MGS} \qquad (1)$$

$$\mathrm{XAP} + \mathrm{MGS} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\mathrm{XAP}\cdots\mathrm{MGS}] \xrightarrow{k_2} \mathrm{XAP\text{-}MGS} \qquad (2)$$

The first is that the reaction follows a simple biomolecular process. In the second case, the inactivator first combines with the enzyme in a reversible fashion forming an EI complex. Subsequently, a second step, k_2, causes the irreversible covalent inactivation of the enzyme. In this case, the rate saturation effects are entirely analogous Michaelis-Menten treatment of substrate binding. The 2 mechanisms can be distinguished by measuring the inactivation half-life (τ) at various concentrations of the inactivator (I). If the inactivation is a simpler bimolecular reaction as represented in equation (1), the following relationship is obtained:

$$\tau = \frac{\ln 2}{[I]\, k_{2nd}}$$

in which τ is the half-time of inactivation, and [I] is the molar concentration of the inhibitor (Aldridge, 1950). On the other hand, if the inhibitor combines reversibly with the active site before covalent modification as shown in equation (2), applying steady state kinetics, Meloche (1967) has derived a linear rate expression corresponding to equation (2) which shows the dependence of the inactivation rate on inhibitor concentration:

$$\tau = \frac{1}{[I]} (T K_{inact}) + T.$$

K_{inact} is $\frac{k_{-1} + k_2}{k_1}$ and is comparable to K_m in the Briggs-Haldane treatment of the Michaelis-Menton expression, and T is the finite minimal inactivation half-time. Since a plot of τ *vs.* $\frac{1}{[I]}$ should intercept the τ axis at zero if equation (1) is operative, and at a finite value (T) if equation (2) is operative, the 2 schemes are kinetically distinguishable. Figure 2 shows the plot of τ *vs.* $\frac{1}{[XAP]mM}$. Limiting half-lives of inactivation of 52 and 13 min were obtained for the bromo- and iodoacetol phosphates, respectively. These results suggest that the inactivation mechanism follows the second scheme [equation (2)], which is a typical course of inactivation mechanism for competitive types of inhibitors. Furthermore, apparent dissociation constants of 0.25 and 1.1 mM obtained for the bromo- and iodoacetol phosphates, respectively, compare well with the value of 0.20 mM for the K_m for dihydroxyacetone phosphate.

In order to gain further insight as to the nature of this nucleophilic residue in the active center, the enzyme was inactivated with iodoacetol phosphate, then treated with [^{3}H]$NaBH_4$ (706 mCi/mmole) for 30 min, at 30 C, pH 8 (0.1 mM $NAHCO_3$ buffer), thereby reducing the carbonyl of the incorporated reagent and introducing a tritium label (Hartman, 1971). The reaction mixture was then made 0.1 M in β-mercaptoethanol and dialyzed exhaustively against water for 7 days. A net radioactivity incorporated to the protein was 6 x 10^5 cpm (after subtracting a "control" in which native methylglyoxal synthase was treated under the same condition, but without haloacetol phosphate). This tritium labeled sample was divided into 3 aliquots and subjected to acid hydrolysis for 24, 48 and 72 hrs, followed by automatic amino acid analysis. The column eluant was collected with a fraction collector at a rate of 1 min/fraction from the outlet of a fluorometer. A single radioactive peak which was fluorescence-negative appeared 9 min ahead of the first fluorescence-positive amino acid residue--aspartic acid (Fig. 3). The identity of this radioactive derivative would appear to be the result of a modification of either an aspartic or glutamic acid residue in the active center. This finding is consistent with the results found in hydrolysates of triosephosphate isomerase which had been treated with chloroacetol phosphate and NaB^3H_4 (Hartman, 1971). This *o*-phthalaldehyde negative species was found to be glycerol, which was formed during acid hydrolysis. This indicates that the original modification occurred through an acid labile linkage. Lin, *et al.*, (1971) showed that the thioether linkage between cysteine and chloroacetol phosphate is not likely to be the case for the modification of methylglyoxal synthase. Furthermore, the possibility that the modification occurred

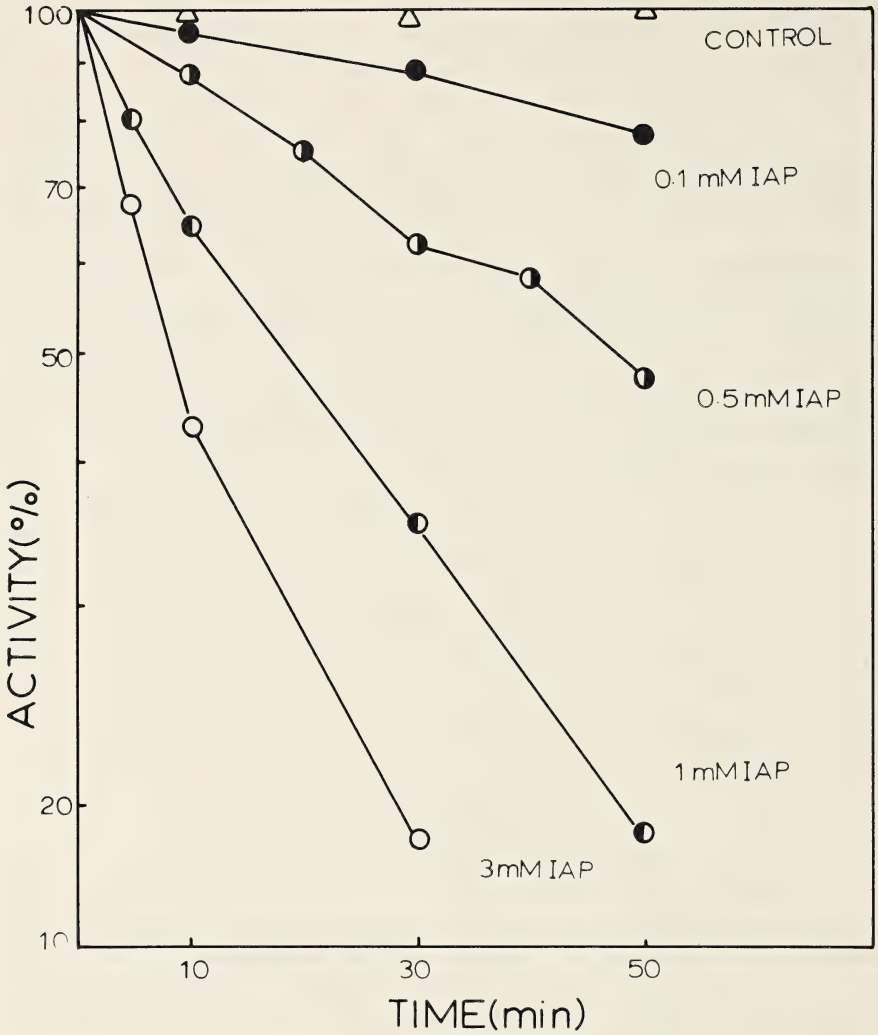
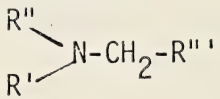


Figure 1. Time course inactivation of methylglyoxal by iodoacetol phosphate (IAP). Methylglyoxal synthase (0.1 mg/ml) was incubated with 0.1 mM IAP (●), 0.5 mM IAP (◐) 1 mM IAP (◑), 3 mM IAP (○), and 0.0 mM IAP (△) at 37 C, pH 7.0 (50 mM imidazole). Periodically, samples of the reaction mixture were withdrawn and assayed immediately by the coupled enzyme spectrophotometric method.

between a basic amino acid residue such as histidine, arginine, or lysine can be also precluded since derivatives of the type,



were shown to be stable during acid hydrolysis of modified phosphoglucose isomerase (Gibson, *et al.*, 1977). Therefore, the most likely modification would seem to be the formation of an ester derivative of glutamate or aspartate.

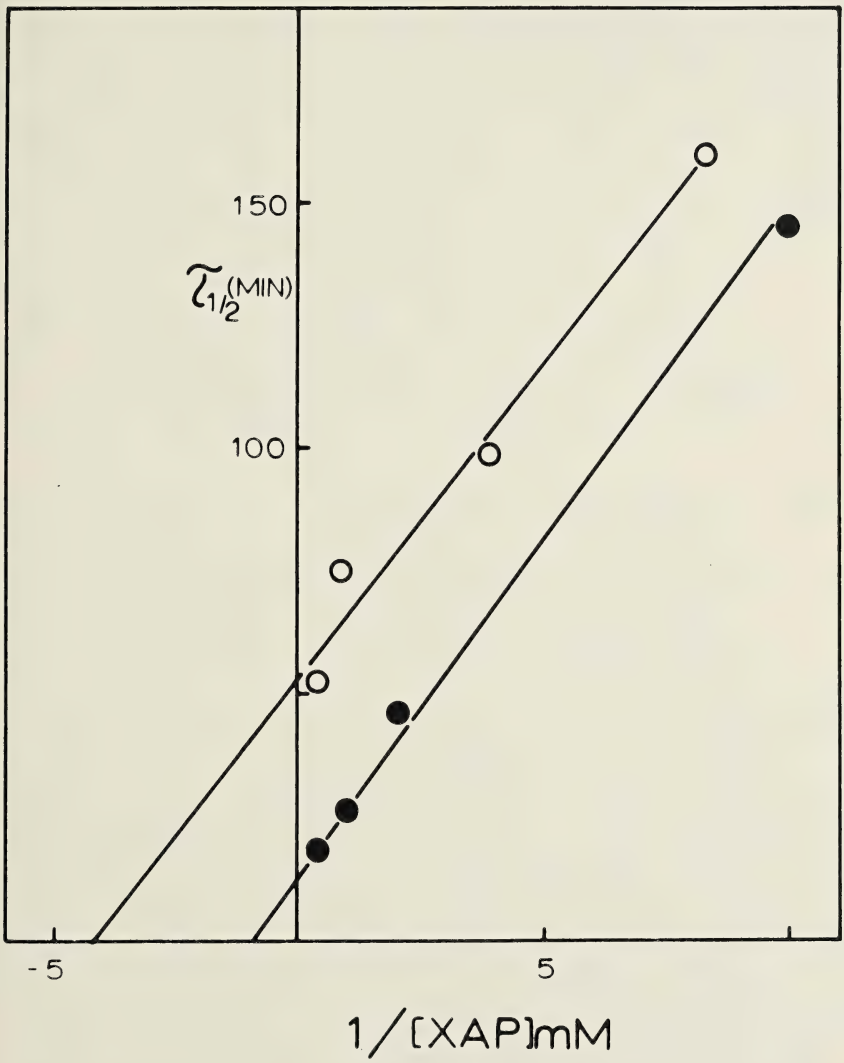


Figure 2. Inhibition half-life ($\tau_{1/2}$) determined from the initial rates of inactivation as a function of the reciprocal of concentrations of bromoacetol phosphate (0) or iodoacetol phosphate (●). Reaction mixtures buffered with 50 mM imidazole (pH 7.0) contained methylglyoxal synthase (0.1 mg/ml), and various concentrations of bromo- and iodoactol phosphate at 37 C.

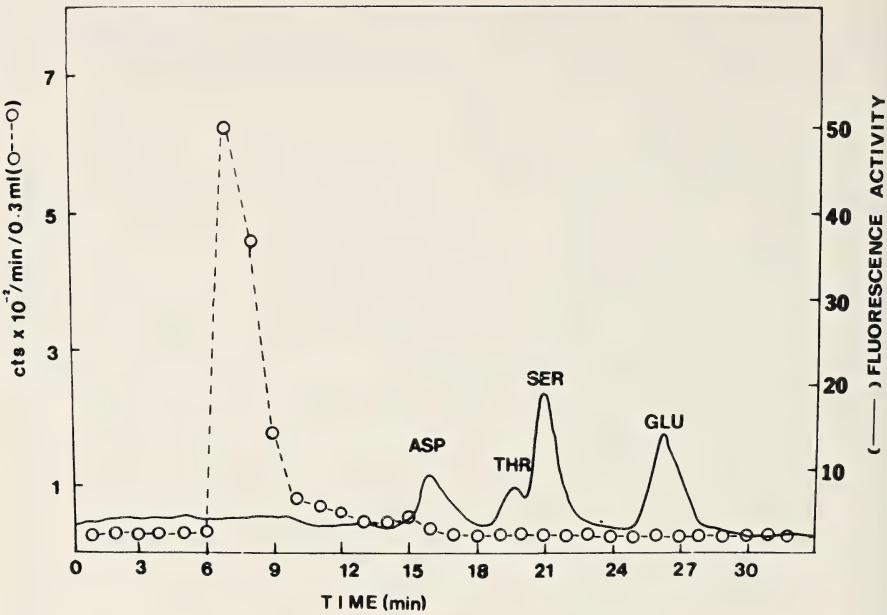


Figure 3. Partial chromatogram of the acid hydrolysate of methylglyoxal synthase after modification with iodoacetol phosphate, followed by reduction with [^{3}H] $NaBH_4$. The fluorescence pattern (solid line) was directly traced from the recording chart paper. Radioactivity of eluant fractions (open symbols) were counted in a liquid scintillation spectrophotometer. Only the first portion of the chromatogram is shown, although the entire eluate was analyzed. No additional radioactive peaks were detected.

The substrate, dihydroxyacetone phosphate, exists in neutral aqueous solution at 20 C as a mixture of keto, gem diol and enolic forms in the ratio 55:44:1 (Reynolds, *et al.*, 1971). While it is not known which of these forms of dihydroxyacetone phosphate bind, the enol is known to exist in relatively strong alkaline solution (Sellinger, *et al.*, 1958). Furthermore, the formation of the hydrogen-bonded enol has been shown to facilitate the phosphoester hydrolysis (Sellinger, *et al.*, 1958).

From the above considerations and observations, a possible reaction mechanism is shown in Fig. 4. In this scheme, methylglyoxal synthase, like triosephosphate isomerase, α-glycerophosphate dehydrogenase and aldolase, is shown as binding the keto form of dihydroxyacetone phosphate as the active substrate. However, this does not preclude the binding of the other tautomeric forms of substrate. A carboxylate group from either glutamic or aspartic acids located in the active center first catalyzes the keto-enol tautomerization to the hydrogen-bonded enol form. Since neither a metal nor Schiff's base appears to be involved in polarizing the carbonyl of dihydroxyacetone phosphate, it is likely that a

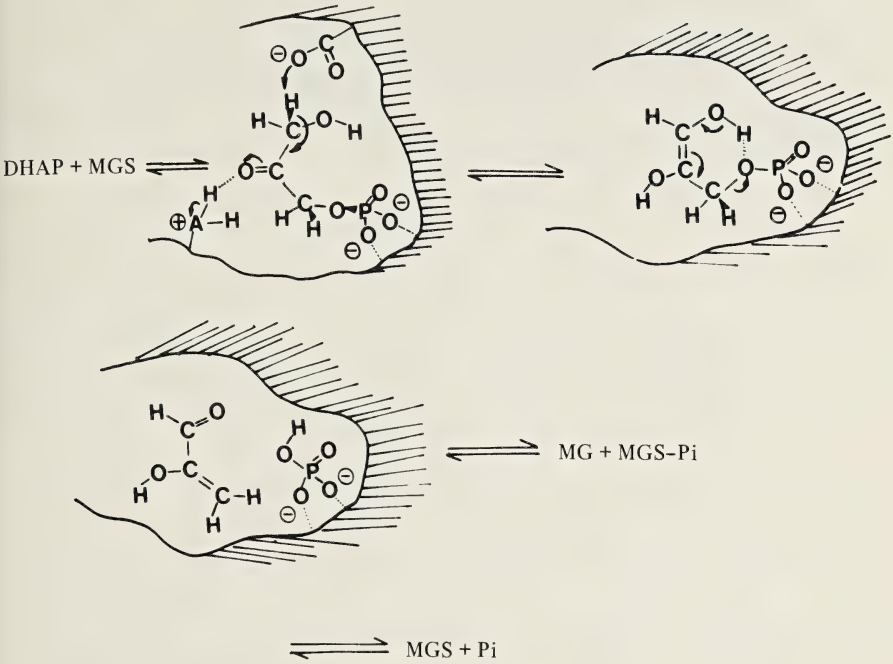


Figure 4. Proposed mechanism of catalytic action of methylglyoxal synthase.

proton derived from an electrophilic amino acid residue is involved in the formation of the enediol. The hydrogen-bonded enediol intermediate then facilitates the oxidation-reduction and phosphoester cleavage with C-O bond breaking, followed by the ordered release of methylglyoxal prior to phosphate. The proposed mechanism also indicates requisite phosphate binding domain.

Recently, Summers and Rose (1977) have considered the stereochemistry of the deprotonation and protonation in this reaction by using partially purified methylglyoxal synthase from *E. coli*. Their studies indicate that the enzyme stereospecifically abstracts the pro-S hydrogen at C-3 of dihydroxyacetone phosphate and nonstereospecifically protonates the C-3 methyl group of methylglyoxal. Thus, these observations further corroborate the proposed mechanism.

The proposed reaction scheme incorporates and is consistent with all the present known information on the enzyme. Future studies such as the identification of the electrophoretic and nucleophilic groups in the active center, the composition and sequence of the active-site region of the enzyme, will be necessary to further verify and extend our knowledge of this unusual enzyme. Perhaps only with full understanding of the enzyme itself will we be able to fully appreciate the physiological significance of the enzyme and its role in the metabolism of methylglyoxal.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institute of Arthritis, Metabolic and Digestive Diseases (AM14638), a Multidisciplinary Center Grant from the Administration on Aging (90-A-1050), the Robert A. Welch Foundation (B-502), and NTSU Faculty Research Funds.

COMMENTS

Gauntt: Is there any evidence for the existence of this pathway in mammalian cells?

Gracy: This is a particularly interesting question, and, in fact, precisely why we initially began these studies. For several years we have been interested in the genetically inherited triosephosphate isomerase deficiency disease. This disorder causes an extremely severe neuromuscular disorder with accompanying developmental retardation and chronic hemolytic anemia. It had been reported several years ago that in patients with this genetic disease, levels of dihydroxyacetone phosphate were markedly elevated. However, upon administration of ^{14}C-labeled glucose to erythrocytes from the patient, there was still a stoichiometry of 2 equivalents of pyruvate formed/glucose. Moreover, massive doses of glucose did not further increase the pool size of dihydroxyacetone phosphate. Since we knew that the erythrocyte has an active glyoxalase I and II system, we became quite interested when the methylglyoxal synthase was first reported in *E. coli*. If a similar enzyme existed in erythrocytes, it could explain these metabolic observations in the patient with triosephosphate isomerase deficiency. Moreover, the highly toxic methylglyoxal looked like a very good candidate for the ultimate product which causes cell and tissue damage in these individuals. Thus, we launched a major effort to examine a variety of human and other mammalian tissues for the presence of the enzyme. While this might initially seem like a simple task, it was complicated by the possibility that when one adds dihydroxyacetone phosphate to a crude cell homogenate, it is very likely rapidly catabolized by a number of highly active enzymes with high affinity for the substrate (e.g., triosephosphate isomerase, aldolase, alpha glycerophosphate dehydrogenase, phosphatases, etc.). We developed several methods of assaying the enzyme and have surveyed bacteria, fungi, yeast, a variety of invertebrates and vertebrates, and the only source of the enzyme known thus far is some of the enteric bacteria.

Gauntt: Is this enzyme present in enteropathogenic *E. coli*?

Gracy: We have not examined this. However, it is possible that enteric bacteria could produce toxic levels of methylglyoxal. It is known that under certain growth conditions *E. coli* will excrete relatively high levels of methylglyoxal in culture media.

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RELEASE OF TEMPERATURE-SENSITIVE DNA-BINDING PROTEINS FROM FOLDED NUCLEOIDS OF AN *ESCHERICHIA COLI* *dnaA*^{ts} MUTANT

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ABSTRACT

DNA-binding proteins which have been preferentially released at 43 C from the folded chromosomes of an *E. coli dnaA*^{ts} mutant have been examined. SDS-polyacrylamide gel electrophoresis analysis reveals the release of proteins whose polypeptide constituents may be categorized into 3 molecular weight ranges: 40, 90 and 150-160 kD. These molecular weights correspond well with those of the α, σ, β and β' subunits of the *E. coli* K12 DNA-dependent RNA polymerase suggesting a possible interaction between the *dnaA* gene product and the RNA polymerase.

INTRODUCTION

The isolation of conditionally-lethal, temperature-sensitive initiation mutants in *E. coli* has confirmed the role of specific initiation proteins in the process of initiation of chromosome replication. When placed at a non-permissive temperature these mutants are capable of finishing the ongoing round of DNA synthesis but cannot initiate new cycles of replication (Hirota, *et al.*, 1968; Hirota, *et al.*, 1971; Gross, 1971; Beyersmann, *et al.*, 1974). Of the 13 known genetically distinct *dna* mutations only 4 are specifically defective in the initiation process. These include *dnaA, dnaC, dnaI,* and *dnaP* (Geider, 1976). Although the *dnaC* gene product has been partially purified (Wickner, *et al.*, 1974) the actual role of any of these gene products in the initiation process remains unknown.

Perhaps one of the best characterized mutants of this group is the *dnaA*^{ts} mutation (Abe and Tomizawa, 1971); Wechsler and Gross, 1971; Hirota, *et al.*, 1970). Genetic studies have shown the gene product of the *dnaA* locus to be a trans-acting protein (Beyersmann, *et al.*, 1974) which may be multimeric (Gotfried and Wechsler, 1977) and may interact with other multimeric proteins in a complex essential for the initiation and replication of the bacterial chromosome (Wickner, *et al.*, 1974; Scheckman, *et al.*, 1975). The involvement of the bacterial

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membrane in the role of the *dnaA* gene product has also been suggested (Fralick and Lark, 1973; Wechsler and Zdzienicka, 1975). The *dnaA* gene product appears to act early in the sequence of events leading to initiation (Zyskind, *et al.*, 1977) and it has been suggested that the *dnaA* gene product is intimately involved with the synthesis of origin RNA-polynucleotide primers (O-RNA) (Messer, *et al.*, 1975; Zyskind and Smith, 1977). Recently, investigators have mapped extragenic suppressors of the $dnaA^{ts}$ phenotype within the *rpoB* cistron, implicating interaction of the *dnaA* gene product with RNA polymerase (Bagdasarian, *et al.*, 1977). Physiological investigations in our laboratory could be interpreted to support this idea.

MATERIALS AND METHODS

Bacterial Strains

E. coli N167 is a $dnaA^{ts}$ mutant. HMT is the $dnaA^{+}$ parent of N167. The restrictive temperature for initiation of N167 is 43 C (Abe and Tomizawa, 1971).

Media and Growth Conditions

Cells were grown at 30 C in 160 ml of minimal-media A (Miller, 1972) with 0.05% casamino acids, and 10 μCi/ml ^{3}H-amino acids with a specific activity of 62.2 μCi/μg (ICN). At 10^{8} cells/ml the cells were washed by filtration and harvested by centrifugation using a Beckman JA-20 rotor at 2500 x g (R_{av}) for 5 min.

Isolation and Treatment of Nucleoids

Folded nucleoids were isolated from freshly harvested cells following lysis at 25 C by the method of Kornberg, *et al.*, 1974 (Fig. 1). Nucleoids were then suspended in elution buffer (Eaton, *et al.*, 1977) and incubated at either 30 C or 43 C for 15 min. The nucleoids were sedimented immediately in a Brinkmann 3200 microcentrifuge for 10 min at 8,000 x g (R_{av}) and the supernatants were collected by pipette. A general outline of the procedure is illustrated in Fig. 2.

Polyacrylamide Gel Electrophoresis

Supernatant samples were dialyzed against 0.1% SDS, 0.1M TRIS pH 6.8 and 1.0% BMSH at 25 C overnight. Each sample was quick-frozen with dry ice and lyophilized with a Freeze-Dry 3 lyophilizer (Lab Con Co.). Samples were taken up in 200 μl of the dialysis buffer and boiled for 5 min (Lugtenberg, *et al.*, 1975) at a ratio of 1 μg SDS/1μg protein.

The samples were applied to 10% polyacrylamide disc gels (Studier and Maizel, 1969; Weber and Osborn, 1969) which were run at 1.5 mAmp/tube for 4 to 5 hrs. The gels were either stained, or quick-frozen and serially sliced into 1 mm fractions with a Mickle gel slicer. Each slice was solubilized at 41 C overnight in 0.5 ml of 30% H_2O_2 made alkaline with 1:50 volume of concentrated NH_4OH

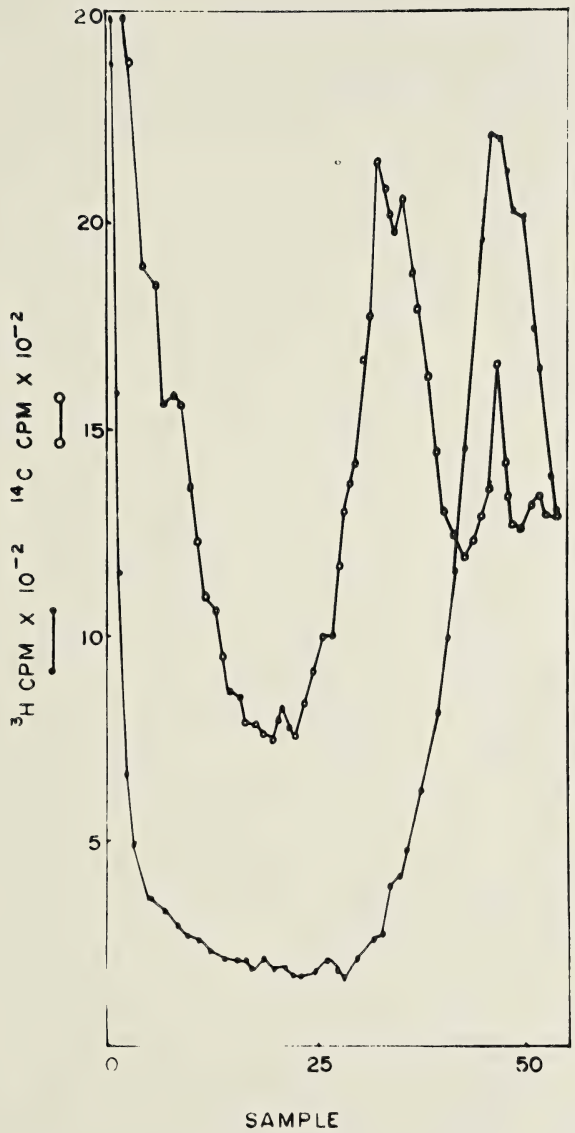

Figure 1. 12-60% sucrose gradient sedimentation profile of folded nucleoids isolated at 43 C compared with sedimentation profiles of whole cells and sheared DNA. ^{14}C-thymine labelled folded nucleoids (0–0) and ^{3}H-thymidine labelled whole cells and sheared DNA (prepared from isolated nucleoids) (●–●) were mixed and placed on the same 12-60% sucrose gradient and centrifuged (vide infra). Note the whole cells in the bottom of the gradient, the sheared DNA on the top of the gradient, and the folded nucleoids in a distinctly separate peak near the center of the gradient.

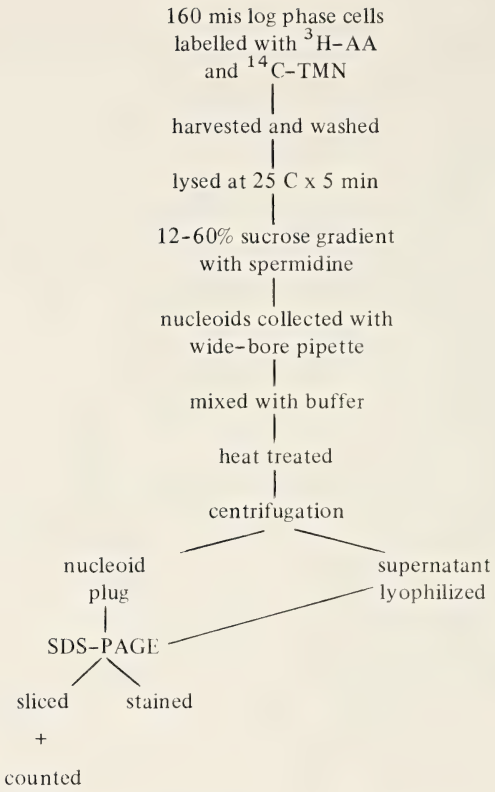

Figure 2. Generalized outline of folded nucleod isolation procedure.

(Lastick and McConkey, 1976). The solubilized samples were taken up in 3.5 ml of Triton X-114-xylene scintillation cocktail, gently mixed and counted on a Beckman LS-230 scintillation counter.

RESULTS AND DISCUSSION

Initially, *E. coli* temperature-sensitive DNA-binding proteins were fractioned *in vitro* by DNA-cellulose column chromatography. Polypeptides suggestive of the β and β' subunits of RNA polymerase were present in the 43 C eluted proteins of the *dnaA* mutant examined, but were not present in the 43 C eluted proteins of either the *dnaA*$^{+}$ parent strains, or a *dnaC* mutant. This suggested a specific temperature release of RNA polymerase in the *dnaA* mutant (Eaton, *et al.*, 1977).

Subsequently, we have examined the *in vitro* temperature mediated release of RNA polymerase from folded nucleoids of a *dnaA167* mutant, and its *dnaA*$^{+}$ parent, HMT.

At 43 C *dnaA167* nucleoids show preferential release of polypeptides in the molecular weight regions of 90, and 150-160kD when compared with the same nucleoids incubated at 30 C (Fig. 3). However, at 43 C, *dnaA167* nucleoids show preferential release of polypeptides in the molecular weight regions of 40, 90 and 150-160kD when compared with proteins released at 43 C from HMT ($dnaA^+$) nucleoids (Fig. 4).

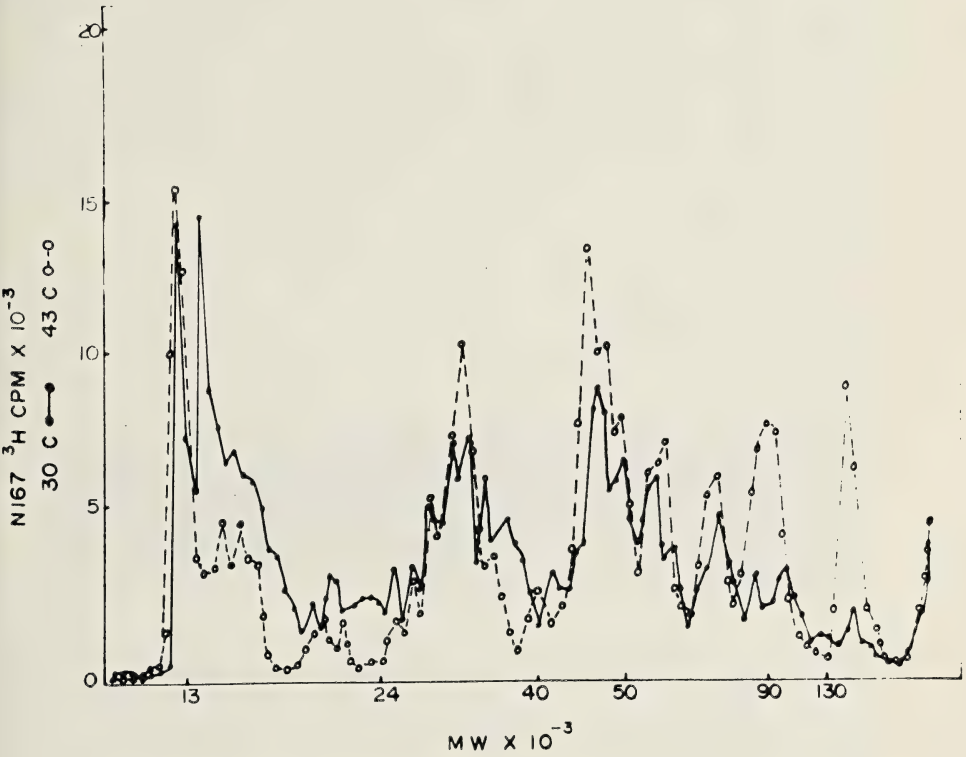

Figure 3. Comparison of proteins released at 30 C and 43 C from N167 ($dnaA^{ts}$) nucleoids. Comparison of serially sliced gels representing the radioactive profiles of proteins released from N167 ($dnaA^{ts}$) folded nucleoids at 30 C (●—●) and 43 C (o--o). Note the potentiation of the peaks in the 43 C supernatant sample, particularly at 90 kD and above 130 kD. Electrophoresis (as in all figures following) is from right to left. Molecular weight standards are: Chymotrypsin, 13 kD; apoferritin principle polypeptide, 24 kD; gamma globulin heavy chain, 50 kD; beta-galactosidase principle polypeptide, 130 kD; RNA-polymerase α subunit, 40 kD and RNA-polymerase σ subunit, 90 kD.

These molecular weight ranges are identical with those of the α, σ, β, and β' polypeptides of a known *E. coli* K12 DNA-dependent RNA polymerase (Lewin, 1974).

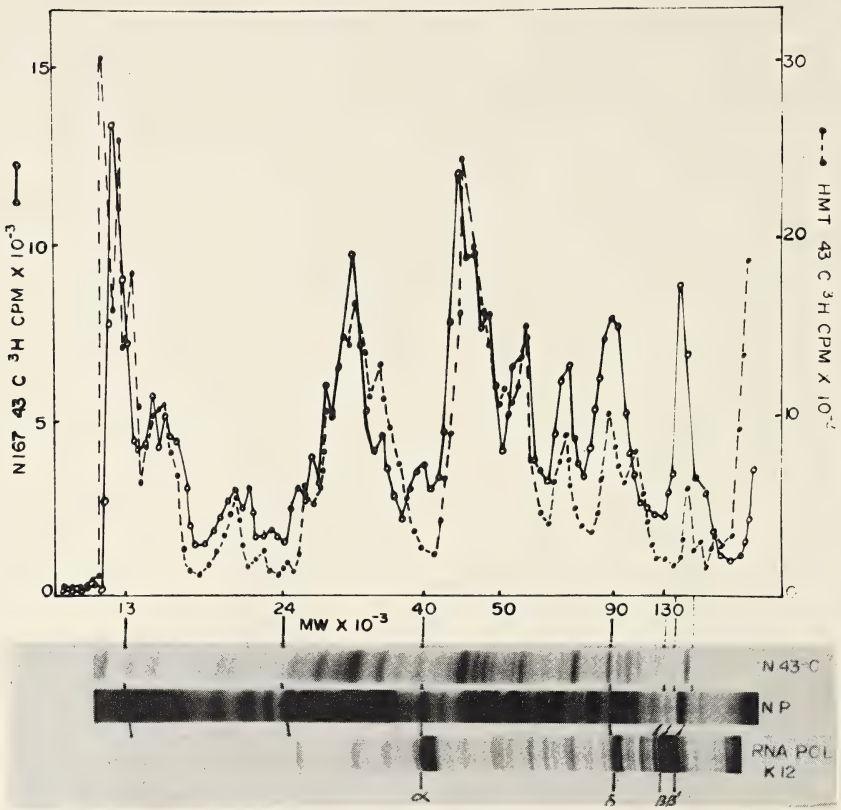

Figure 4. Comparison of sliced and stained polyacrylamide gels of HMT ($dnaA^+$) and N167 (dna^{ts}) 43 C released proteins. Comparison of sliced gel profiles of 43 C released proteins of HMT (●--●) and 43 C released proteins of N167 (0–0). Note the potentiation of peaks in the 43 C N167 profile, particularly at 40 kD, and 90 kD and above 130 kD. Compare these with stained gels of 43 C released proteins of N167 (43 C) (the same sample for which a sliced gel profile is presented above); proteins remaining with the nucleoids after temperature treatment (NP–nucleoid proteins) and a comparison gel of a known K12 RNA polymerase (RNA POL K12). It is apparent that the potentiated peaks at 40 kD, 90 kD and approximately 150-160 kD coincide with the known RNA-polymerase subunits. That the band in the ββ' region of the 43 C released N167 nucleoid protein sample above is really 2 closely migrating bands (as β and β' should be) was confirmed by densitometry scan in conjunction with a Du Pont 310 electronic curve analyzer analysis.

In a series of experiments we find this enhanced release to be 2-5 times that of the control values. Assuming that the 150-160kD peak is composed only of β and β' subunits, this would represent release of > 10% of the RNA polymerase present in the nucleoid sample.

A trivial explanation for this enhanced release would be selective unfolding of the *dnaA167* nucleoids at 43 C, resulting in the release of RNA polymerase. Using mixtures of ^{3}H-labeled N167 and ^{14}C-labeled HMT nucleoids we have shown that the amount of unfolded DNA in the supernatants represents 15-20% of the total nucleoid DNA (possibly due to shearing during pipetting) regardless of strain or temperature. The apparent increase in RNA polymerase release seen in Figs. 3 and 4 therefore must be due to some factor other than the selective unfolding of the N167 nucleoids at 43 C.

We are aware that this amount of apparent RNA polymerase release would not be expected if the *dnaA* gene product acted in a stoichiometric manner with the RNA polymerase. Consequently, the possibilities of some non-specific pleiotropic interaction cannot be ruled out. Experiments designed to resolve this problem are presently in progress.

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APPLICATION OF A PHYSICAL MAPPING METHOD TO VECTORS AND CLONED DNA

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INTRODUCTION

A fundamental aspect of genetics is the need to accurately map the locations of genes and genetic markers. Classically this meant ordering the markers and estimating the relative separation between markers in terms of recombination frequency. With the discovery of DNA as the genetic material it became possible to specify the location of genetic markers with respect to the DNA molecule. By controlled shearing of viral or bacterial DNA and measurement of molecular weights, approximate separation of markers could be determined in terms of nucleotides (Hogness, *et al.*, 1966). Using density-labeled DNA, Jordan and Meselson (1965) demonstrated that the map positions determined by recombination corresponded approximately to those determined physically on phage DNA. More recently electron microscopic procedures have been developed that allow the location of deletions, substitutions, duplications, and regions of non-homology to be accurately determined (Davis, *et al.*, 1971). The location of promoters can now be determined by locating the position of bound RNA polymerase or by determining the start site of mRNA synthesis by measuring the length of nascent RNA molecules still bound to the DNA (Delius, *et al.*, 1973). Operators have been mapped by determining the location of bound repressor on the DNA. Most recently the ultimate mapping procedure, DNA sequencing, has allowed the position of genetic markers to be determined to the exact nucleotide (Maxam and Gilbert, 1977).

We have recently developed an electron microscopic procedure which allows the position of ribosome binding sites to be determined (Calame and Ihler, 1977a). The position of the ribosome marks the beginning of the gene (corresponding to the amino terminus of the protein). The length of the gene can be readily calculated from the molecular weight of the protein as determined on SDS polyacrilimide gels. The resulting map thus indicates the position and extent of the gene and also whether there is untranslated DNA between genes.

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MATERIALS AND METHODS

It is useful to employ an internal marker in the DNA to orient the direction and to eliminate length errors caused by nucleolytic cleavage of the DNA. Many types of markers could be used, but a stem and loop resulting from insertion of a Tn element is convenient. Many different insertions are available. We have used λbb-kan2 (Berg, *et al.*, 1975) which has a Tn5 insertion in *rex* at 75.8% and λ::Tn10 with Tn10 inserted at 53% (Berg, personal communication).

Ribosomes and factors were purified from *E. coli* 238 (DNase I^-, $B1^-$,) by the procedure described by Anderson, *et al.*, (1967). Gene 32 protein was obtained from T4-infected *E. coli* lysates by chromatography on DNA-cellulose (Alberts and Frey, 1970). Binding of the ribosomes, fixation, and preparation for electron microscopy were carried out as described by Ihler, *et al.*, (1978). In brief, ribosomes, factors, f-metRNA, and single-stranded DNA are mixed and incubated and the resulting complex is purified by glycerol gradient sedimentation. Ribosomes are pre-fixed with 0.1% glutaraldehyde, gene 32 protein is added and the entire complex fixed with 0.3% glutaraldehyde. Samples are spread using the cytochrome c-formamide technique following the method of Davis, *et al.*, (1971).

RESULTS AND DISCUSSION

We have found conditions which allow ribosomes to bind to single-stranded DNA specifically at sites corresponding to mRNA translational initiation sites and have developed a procedure by which these bound ribosomes may be visualized in the electron microscope. Use of DNA instead of RNA offers considerable practical advantage for many studies since RNA is in general difficult to isolate in a pure and intact form. Also DNA is more stable and resistant to nucleases than RNA. Most important, an entire genome containing all genes in equimolar amounts can be used. This permits genetic mapping of the ribosome binding sites, easy quantitation of the number of ribosomes bound and concomitant utilization of the many other electron microscopic procedures that have been devised for mapping DNA.

Vectors and Cloned DNA

The application of this procedure to cloned DNA, carried either on plasmids or phage DNA as vectors is obvious and can be important for many reasons including the following:

1. Current guidelines on recombinant DNA require containment of cloned DNA which has not been completely defined as harmless. Since it is difficult to be confident that all polypeptide products of a given fragment have been detected, the possibility may frequently exist that a DNA fragment contains genes which are silent as a result of the absence of a functional promoter. Determination of

the number and location of genes by ribosome binding would be invaluable in deciding whether the possible danger attributable to release of any given fragment can be properly assessed.

2. Clones are selected for specific functions, especially those which can complement a missing bacterial function. The contiguous DNA needs to be defined in relationship to nearby promoters and binding sites so that the resulting polypeptide synthesis can be understood within the framework of regulation.

3. Eucaryotic genes may or may not have binding sites recognizable by *E. coli* ribosomes. The binding map, however, predicts the allowable number of sites for initiation. If a particular eucaryotic initiation site is not recognized by *E. coli* ribosomes, it should be simple to try ribosomes from a number of different sources such as various bacterial strains, yeast, wheat germ and so forth to see which will function to translate the protein.

4. Maps obtained from ordering restriction fragments often are at least partially undefined in terms of which fragment carries which ribosome binding sites, especially where a genotypic assay is not available for all genes present. By our procedure a map showing the position and size of all genes present on a fragment is obtained whether or not mutants in such genes are known. The map obtained can be correlated with whatever other information is available such as genetic recombination data or polypeptide size.

In addition, the vectors need to be genetically better defined since the vector and the cloned DNA coexist on the same DNA molecule. We are currently trying to map the entire lambda genome. We have so far mapped the region between 63% and 86% lambda. Binding sites for all the 12 known genes in this region were found as well as several sites which probably indicate the position of unmapped genes or genes not yet identified genetically (Calame and Ihler, 1977b).

The region from 86%-100% lambda can be mapped using the right end as origin and Tn5 to define the right-left direction or by using lambda with Tn insertions very near the right end (95% or 99%). The *b2* region and the remaining part of the leftward operon can be mapped using Tn10 inserted at 53% lambda. The function of the proteins coded for by the *b2* region are not known, but Hendrix (1971) has shown that this region produces at least 7 proteins synthesized early after infection and 2 synthesized late after infection. The crude map available puts 3 of the early proteins, bEa38, bEa40, and bEa43 at the same site and 3 others, bEa30, bEa32 and bEa56 also at a single site. It is of interest to determine the location, spacing, and strandedness of the initiation sites in this region. The region to the right of *b2* (*int xis red X red B gam*) is also of considerable interest because of the enzymology and genetics of both integration and recombination. In addition, a promoter (pI) located between *int* and *red* (Shimada and Campbell, 1974) is not well characterized. Its function might be better understood

after a good physical map is available for this region. Delius, *et al.*, (1973) devised a procedure by which nascent RNA chains can be visualized and measured, allowing the number and location of the promoters to be determined as well as the direction of transcription. In combination with the ribosome binding site map this information provides a powerful tool for analyzing promoters and operons such as pI.

Much of the left half of lambda can be mapped using Tn10 and the left end of the DNA as origins. Since these genes are essential for formation of lambda structural proteins, insertions in this region would render the phage defective. This author knows of no available insertions in this region to assist in the mapping.

Mapping of plasmids is also of great interest. For example Ippen-Ihler, *et al.*, (1972) determined the genetic map of the transfer genes of F by deletion mapping. Further work in her laboratory identified the products of several of these genes (*traJ*, *traA*, and *traT*) and molecular weights were obtained (Minkley, *et al.*, 1976; Minkley and Ippen-Ihler, 1977). The molecular weights of several other proteins has subsequently been obtained by Kennedy, *et al.*, (1977). An approximate map has been constructed based on EcoRI sites, but a more sophisticated map, including the binding sites for genes not yet identified genetically, would be very helpful.

Mapping of transposable genetic elements (transposons) is also possible. We have mapped Tn10 using λ::Tn10 and have found a total of 8 binding sites, all on the same DNA strand, located at positions 9%, 19%, 23%, 27%, 31%, 46%, 62% and 97% of the total 6.7 kb DNA (Calame and Ihler, unpublished data). From these data the maximum polypeptide sizes could be calculated. They range from 9,500 daltons to 81,000 daltons.

Translational Control

A process as important as protein synthesis is unlikely to occur without numerous kinds of metabolic controls. These controls, known and unknown, are usually grouped under the heading of translational control. One category of controls concerns the mechanism by which the overall rate of protein synthesis in the cell is controlled. In bacteria, for example, the presence of an uncharged tRNA species in the A site (due, for example, to starvation for a required amino acid) triggers the synthesis of ppGpp which in turn shuts off synthesis of rRNA and hence ribosomes. In reticulocytes, hemoglobin synthesis depends on the availability of hemin. In its absence a protein kinase phophorylates IF-E2 (and perhaps other proteins) and protein synthesis shuts off. These forms of control are relatively non-specific and do not allow for the differential translation of different messengers.

In *E. coli* there are several observations which appear to indicate the existence of a more specific translational mechanism to control the amounts of specific proteins synthesized. Although tentative, the conclusion is that different proteins can be synthesized in different amounts despite the respective messengers being

present in equimolar amounts. For example, the late genes of bacteriophage lambda are transcribed from a single promoter and so the genes are transcribed in equal amounts. The levels of mRNA for different genes, both total RNA and functional RNA, do not vary more than 2-fold. The molar ratios of the protein products however vary almost 1000-fold (Ray and Pearson, 1974, 1975). A similar argument can be made for the lambda early genes as well (Hendrix, 1971).

In the *lac* operon, β-galactosidase monomer polypeptides are synthesized in a ratio of 3.5-7:1 as compared to transacetylase and this ratio may vary depending on growth conditions (Zabin and Fowler, 1970). On the other hand enzymes of the tryptophan operon (Morse, *et al.*, 1968) and the galactose operon (Wilson and Hogness, 1969) are synthesized in equimolar amounts. Kennell and Riezman (1977) have made detailed calculations on the *lac* operon mRNA and concluded that ribosomes initiate β-galactosidase at 3.2 second intervals and initiate galactoside acetyltransferase at 16 second intervals.

Although a variety of mechanisms could be responsible for the synthesis of differing molar ratios of polypeptides, an attractive but unproven mechanism is that the sequence of nucleotides at or near the initiation site controls the intrinsic translational rate. This could be imagined to occur either through the binding affinity of the ribosome for the site or through the ease or difficulty of initiating polypeptide synthesis by joining the first polypeptide bonds.

The mechanism by which initiation sites are selected is not fully understood. One important element is the initiator codon. Polypeptides in *E. coli* are initially synthesized with a N-terminal methionine derived from a particular species of charged tRNA, f-met-$tRNA_f^{met}$ which recognizes as its codon AUG or GUG. Under non-physiological conditions *in vitro*, ribosomes can synthesize polypeptides using RNA which lacks the initiator AUG or GUG codons, but an AUG or GUG codon is required for specific initiation and one is present at all natural initiation sites. Even in synthetic RNA, the presence of an AUG will determine the reading frame (Stanley, *et al.*, 1966). An AUG or GUG trinucleotide is sufficient to direct the binding of f-met-$tRNA_f^{met}$ to ribosomes (Clark and Marcker, 1966).

The sequences of many initiation regions are now known, including all of the sequences used by φX174 (Sanger, *et al.*, 1977). No two of these sequences are identical. All contain an AUG or GUG. Preceding the initiation codon by a variable distance of about 10 nucleotides is a region of up to 9 nucleotides complementary to part of the sequence of nucleotides from the 3' end of *E. coli* 16S RNA, ($_{3'}$AUUCCUCCACUAG..$_{5'}$). From 2-5 of the CCUCC rRNA bases may be involved in the complementary region. The λ *cro* sequence (UAAGGAGGU..., Steege, 1977) is complementary to the entire nine base sequence of the 3' terminus. Shine and Dalgarno (1974) proposed that pairing between mRNA and rRNA occurs as part of the initiation process. Steitz and Jakes (1975) demonstrated the existence of this base paired RNA:RNA duplex in initiation complexes. An attractive but unproven hypothesis suggests that the degree of base sequence

homology would help determine the initiation frequency. It could be argued that high levels of homology could promote binding to the site by base pair interaction and lead to high levels of synthesis. Alternatively it could be argued that it might be difficult to disrupt the base pairing, leading to a slow rate of initiation. Homology could in principle either promote or discourage high levels of initiation.

Examination of known mRNA sequences shows that many internal AUG and GUG sequences have sequences homologous to the 3' end of rRNA located upstream from the AUG. It is hard to account for the observed inactivity of these sites for polypeptide synthesis except by assuming either that there is a third unknown factor operating for site selection or that those sites are masked by secondary structure. I do not favor the idea that secondary structure acts in the majority of cases to restrict binding, but it is clear that in some cases this occurs. For example, Qβ replicase protein is synthesized only after part of the upstream coat protein cistron is translated. Sequence analysis suggests that the replicase initiation site is hydrogen-bonded to this part of the coat protein sequence. One of the T7 cistrons (0.3) requires prior RNase III cleavage before it can be efficiently translated, suggesting that this initiation site may be masked by secondary structure (Dunn and Studier, 1975). However, none of the other early T7 cistrons require RNase III processing for protein synthesis to occur.

Despite intensive investigation, the role of initiation factors in site selection is still not fully understood. IF-3 has a role in the dissociation of 70S ribosomes into subunits and also a role in the binding of mRNA to the 30S subunit. It is still not certain that IF-3 plays a direct role in site selection because it can stimulate translation of synthetic polynucleotides as well as natural mRNA. However, if IF-3 has 2 distinct roles (as opposed to 1 role with 2 measurable effects) its effect on synthetic mRNA translation may be a reflection of, for example, its dissociation factor activity. IF-1 and IF-2 are required for the binding of the initiator f-met-tRNA$_f^{met}$ and IF-2 can directly bind the initiator tRNA (Miller and Wahba, 1973). Probably neither has a role in site selection beyond that played by the tRNA.

Protein S1, a 30S protein, is found in molar amounts on active ribosomes and is required for effective initiation (van Duin and van Knippenberg, 1974). It has a high affinity for RNA and if present in excessive amounts it can inhibit translation (Groner, *et al.*, 1972). S1 is not required for binding of f-met-tRNA as directed by AUG trinucleotides, but is required for binding to natural mRNA. It appears to be located adjacent to the 3' end of 16S RNA (Kenner, 1973, Czernilofsky, *et al.*, 1975). Some experiments indicate that S1 may bind strongly to the 3' terminus of the 16S RNA. Dahlberg and Dahlberg (1975) suggested that it may act by destabilizing the hydrogen-bonded bases near the 3' end of the rRNA, facilitating pairing with the homologous mRNA sequences.

S1 may play an important role in correct recognition of the initiation site. *B. stearothermophilus* ribosomes are unable to translate the f2 coat and replicase

cistrons, but are able to synthesize the A protein. The 30S ribosome is responsible for this specificity difference, but not the 3' end of the 16S rRNA since 3' sequence of *B. stearothermophilus* 16S rRNA is identical to the *E. coli* sequence except that there is a sequence $UCUA_3$, instead of the terminal 3'A. Reconstitution experiments indicate that proteins are responsible for the specificity difference (Goldberg and Steitz, 1974). Isono and Isono (1975) showed that addition of *E. coli* S1 to *B. stearothermophilus* ribosomes permitted translation of the f2 coat and replicase proteins. *B. stearothermophilus* ribosomes appear to lack the S1 protein (Isono and Isono, 1976).

The data obtained so far indicates that all the binding sites examined have nearly the same number of ribosomes bound. Table 1(a) shows the data obtained for the number of ribosomes bound to the known lambda genes (Calame and Ihler, 1977b) and the Table 1(b) shows similar data obtained for Tn10 (Calame and Ihler, unpublished results).

TABLE 1

The number of bound ribosomes is taken as a measure of the relative affinity of the ribosome for different sites. Data for the *R* and *L* strands of lambda were determined with both strands present in equal amounts in the binding mixture and so *R* and *L* sites can probably be directly compared. Four of the sites (19%, 23%, 27%, and 31%) on Tn10 do not resolve well. The total bound to all 4 sites are 198 ribosomes, an average of 49/site. The numbers given for Tn10 cannot be compared to the numbers given for lambda since the bound ribosomes were determined in separate experiments.

(A) Lambda				(B) Tn10		
Strand	Position	Presumptive Gene	No. of Bound Ribosomes	Strand	Position	No. of Bound Ribosomes
R	63.8	*Ea22*	14	R	9.0	42
R	64.7	*Ea9*	7	R	19, 23, 27, 31	49 (198)
R	66.3	*exo*	11	R	46.0	42
R	68.0	*bet*	10	R	62.0	42
R	69.1	*gam*	13	R	97.0	41
R	73.3	*N*	12			
R	76.5	*rex*	23			
R	78.2	*cI*	13			
L	78.3	*cro*	16			
L	79.1	*cII*	10			
L	80.5	*O*	12			
L	83.0	*P*	8			

The number of possible binding sites greatly exceeds the number of bound ribosomes (>5:1 in most experiments) so that competition for sites is possible. The binding mixture was incubated sufficiently long that no further net binding occurred. The observed data could result from reactions with rates for the forward reaction sufficiently high for all the sites that the sites appear indistinguishable. In other words, the ribosome might bind to whichever site it first collides. Assuming this might be the case we incubated the bound ribosomes to test whether differential dissociation would be observed. Results entirely similar to those shown in the table were obtained when the complexes isolated on a glycerol gradient were incubated for 10 min at 37 C prior to fixation for electron microscopy. As measured by the millipore filter assay, about 60% of the ribosomes dissociated from the DNA during this incubation.

The simplest interpretation of these results is that all sites have approximately equal affinity for the ribosomes. At first glance, this might seem to be incompatible with the variable amount of complementarity known to exist in the mRNA-rRNA pairing regions. However, this interaction cannot be of prime importance anyway since protein synthesis can take place at temperatures well above the Tm's of the mRNA-rRNA hybrids (Steitz and Jakes, 1975). Additional stabilizing forces are derived from the codon-anticodon pairing of the initiator tRNA and perhaps other tRNA-dependent interactions as well. Initiation factors and S1 may further stabilize the complex.

In lambda there is evidence that the relative amounts of early protein can vary over a wide range, as much as 10-fold to 100-fold. The lambda data shown in Table 1 therefore suggest that the mechanism for variable synthesis does not operate through differential affinity of the ribosomes for the binding site. This leaves open the possibility that the mechanism may involve variable rates of polypeptide initiation.

Is There a Change in Translational Specificity After T4-infection?

It seems plausible that there might be a mechanism for altering the translational specificity for different messengers. However, the need for such a mechanism has been questioned since transcriptional control can readily provide for synthesis of new messengers and cessation of synthesis of old messengers. Since most messengers are unstable, rapid control of protein synthesis can be exerted at the DNA level.

A classic instance of the temporal control of protein synthesis is found after infection by a bacterial virus like T4 in which some proteins are synthesized early after infection and others are synthesized only later. This control is clearly mediated largely at the level of transcription. However, several studies have suggested that there may be translational control exerted after T4-infection as well, since ribosomes from infected cells were found to translate late T4 mRNA efficiently but to be relatively inactive on early T4 mRNA or RNA phage RNA (Ihler and Nakada, 1971; Revel, *et al.*, 1970; Lee-Huang and Ochoa, 1971; Hsu and

Weiss, 1969; Dube and Rudland, 1970; Klem, *et al.*, 1970; Steitz, *et al.*, 1970; Schedl, *et al.*, 1970; Singer and Conway, 1973). Most of these reports also indicated that ribosomes from T4-infected cells are less active than those from uninfected cells and the loss of activity is found in the initiation factor fraction.

At that time IF-3 was thought to be required only for natural messengers so it seemed likely that IF-3 somehow helped recognize and select the initiation site. One hypothesis advanced to explain the T4 data was that IF-3 might be heterogeneous, with individual subfractions recognizing different types of initiation sites. No one has found a new T4-specified IF-3 in infected cells. Lee-Huang and Ochoa (1971, 1973) reported that uninfected cells contained at least 2 forms of IF-3 that apparently recognize different initiation sites on mRNA. These were purified nearly to homogeneity. IF-3 α (23,500 daltons) promoted transcription of MS2 RNA and *E. coli* mRNA, but had relatively little activity on late T4 mRNA (Ratio late T4 mRNA:MS2=0.3) IF-3 β (22,500 daltons) had the opposite specificity (Ratio late T4 mRNA:MS2=5.4). The 2 IF-3 activities were apparently very closely related since they have similar molecular weights and cross-react immunologically. To account for the apparent shift in activity after T4-infection, IF-3 α was supposedly inactivated after infection.

Several reports cast doubt on whether there was a change in translational specificity after T4-infection. Goldman and Lodish (1972) were unable to confirm the experiments showing a change in initiation specificity, although they confirmed that ribosomes from T4-infected cells were deficient in initiation factor activity. Synthesis with poly U or formaldehyde-treated messenger was not inhibited. With f2 only 35%–50% as much protein synthesis occurred using factors from T4-infected cells but all 3 of the proteins were synthesized in the normal relative proportions. Goldman and Lodish also reported that translation of late T4 mRNA was also depressed similarly to f2. Spremulli, *et al.*, (1974) found no change in translational specificity after T4-infection and in addition found that the amount of functional IF-3 activity was not depressed after T4-infection. Schiff, *et al.*, (1974) also reported no change in messenger specificity. There has been no further work with the crude system to resolve this conflict.

The situation became more confused when several proteins called interference factors were isolated which seemed to alter the properties of IF-3. iα inhibited translation of the MS2 coat protein cistron (Groner, *et al.*, 1972; Lee-Huang and Ochoa, 1972; Jay and Kaempfer, 1974) but did not inhibit translation of the MS2 synthetase cistron or T4 mRNA as much. Later however, iα and S1 were shown to be identical (Wahba, *et al.*, 1974, Inouye, *et al.*, 1974). Since S1 has now been shown to be required for ribosome activity (although it is inhibitory when present in large amounts due to binding to the messenger), the significance of the interference factors is completely unclear.

There is no genetic evidence for multiple forms of IF-3 and some evidence indicating that there may be only 1 polypeptide product. Springer, *et al.*, (1977a) isolated a mutant with 2 defects - a thermolabile IF-3 and a modified phenylalanyl tRNA synthetase. A transducing lambda phage was isolated which codes

for a 22,000 dalton protein which comigrates with IF-3 and which is precipitated by anti-IF-3 antiserum (Springer, *et al.*, 1977b). A protein with a slightly lower molecular weight also precipitated with antiserum, a situation reminiscent of the IF-3 α and β situation. The authors speculated that the lower molecular weight form may be a proteolytic product of IF-3. All of this probably indicates that there is only 1 gene product for IF-3 and the existence of modifying proteins is not established.

Despite the uncertainty over the possible roles of IF-3 and S1, the DNA binding evidence indicates that some form of translational alteration occurs after T4 infection and suggests that this alteration occurs in the fraction of proteins which can be dissociated from the ribosomes by 1M salt. The data in Table 2 show that factors from uninfected cells promote binding to the T4 light strand (early genes) as well as T7 DNA and MS2 RNA. Factors from infected cells are effective with the T4 heavy strand (late genes) and not with T4 light strand DNA, MS2 RNA or T7 DNA. The data also suggest that there may be some alterations in the ribosomes as well (or alternatively and perhaps more plausibly that some initiation factors remain after the washing process).

TABLE 2

The number of bound ribosomes/T4 strand was determined for T4 light and heavy strands by a millipore filter assay (Ihler and Nakada, 1971).

Nucleic Acid	A*	B*	C*	D*
T4DNA, Light Strand	15.3	5.1	10.0	<0.1
T4DNA, Heavy Strand	2.0	15.1	4.6	8.0

*A. Washed ribosomes from uninfected cells. Initiation factors from uninfected cells.
B. Washed ribosomes from uninfected cells. Initiation factors from infected cells.
C. Washed ribosomes from infected cells. Initiation factors from uninfected cells.
D. Washed ribosomes from infected cells. Initiation factors from infected cells.

The electron microscopic mapping procedure in principle would allow us to determine which genes are affected by this differential binding effect and to determine which genes are recognized by which factors. However, T4 DNA is not suitable for such an analysis since the T4 map is permuted so that ends cannot be used as origins and since there are no suitable internal markers of the stem and loop type available. In addition, the molecular weight of T4 DNA is very large, making it difficult to avoid fragmentation.

Tikhomirova, *et al.*, (1977) have isolated λ-T4 hybrids. So far only late genes (genes 26, 27, 51 or genes 4, 5, and 50) have been cloned, but the lambda genes would serve in place of T4 early genes, at least for the initial experiments. This should serve also as an assay for specific controlling proteins and allow their purification. Possibly the gene controlling this activity might be *regA* since Wiberg, *et al.*, (1973) have proposed that these mutants are defective in translational control.

Direct Synthesis of Polypeptides on Single-Stranded DNA

For many purposes, it is desirable to know the molecular weight and some physical properties of the proteins coded for by specific DNA molecules. For example, new proteins which appear after viral infection might in principle be of host origin, so it is necessary to demonstrate that the virus codes for the protein. Also, comparison of the final polypeptide with the immediate product of translation can reveal processing or post translational modification. And perhaps most important, the polypeptides can be assigned to genes by demonstrating the disappearance or alteration of a specific polypeptides when coded for by the appropriate mutant. At present one must identify *in vivo* synthesized polypeptides or translate mRNA *in vitro*.

A much simpler alternative would be to directly translate the segment of viral DNA, mitochondrial DNA, or recombinant DNA in question. In addition, such a technique would be invaluable in obtaining products of cloned DNA fragments which contain genes that have been separated from products or sites required for their transcription. The possibility that direct translation of DNA can be accomplished is supported by several observations. First, neomycin is not required for the binding reaction (Bretscher, 1969) and the binding reaction occurs at authentic initiation sites. Neomycin is required for extensive polypeptide synthesis and Bretscher found that only short oligopeptides are synthesized in the absence of neomycin. Morgan, Wells and Khorana (1967) showed that the amino acids incorporated in the presence of neomycin are those specified by the genetic code and that the level of miscoding was very low.

Condit, *et al.*, (1973) investigated the dipeptide frequencies found after initiation of DNA. At the time, no information on the correct dipeptide frequencies was available, but they used ϕX174 DNA in one experiment and subsequently ϕX174 has been sequenced. The predicted dipeptides are: A, met-val; B, met-glu; C, met-arg; D, met-ser; E, met-val; F, met-ser; G, met-phe; H, met-phe; J, met-ser. They found for ϕX174 the following dipeptides (in pmoles): met-val, 0.47; met-thr, 0.24; met$^{leu}_{ile}$, 0.19; met-ala, 0.03; met-ser, 0.08; and met-phe, 0.09. They failed to find significant amounts of met-glu (gene B) and met-arg (gene C) and found significant amounts of met-thr and met-$^{leu}_{ile}$ which are not represented amoung ϕX174 genes. Thus they found dipeptides which might have come from 7 of the 9 genes. But 2 of the 3 most common dipeptides do not correspond to any ϕX174 genes.

By filter binding assay, we find between 5 and 10 sites on ϕX174 DNA (Ihler and Nakada, unpublished results) and using high ratios of ribosomes to DNA, we have seen ϕX174 molecules with up to 9 ribosomes bound (Calame and Ihler, unpublished results). Robertson (1975) found that a single ϕX174 (gene G) accounted for more than 50% of the fragments protected from DNase digestion by bound ribosomes. Since gene G product is also the major polypeptide synthesized by ϕX174 mRNA *in vitro*, this suggests that somehow the stability of the complex might be associated with its frequency of translation *in vitro*. However *in vivo* this preference for gene G is not observed.

One possible approach to demonstrate the synthesis of specific proteins would be to synthesize an enzyme. However, if miscoding is a problem (and it is not clear yet whether it will be or not), it may be desirable to synthesize small proteins. For example, if there is a 98% probability of incorporating the correct amino acid at each position, the probability of correctly synthesizing a protein of 50 amino acids (5,000 daltons) is $(0.98)^{50} = 0.36$. However, the probability of synthesizing a protein of 200 amino acids (20,000 daltons) is only $(0.98)^{200} = 0.02$.

Our first approach was to determine if high molecular weight polypeptides can be synthesized. The product directed by T7 DNA had an average molecular weight of 25,000 daltons, but it was very heterogeneous and did not show any individual peaks. Because T7 codes for many proteins, the absence of defined peaks could be a reflection of the presence of multiple species or could be due simple to non-specific synthesis.

The profile directed by ϕX174 gave a relatively sharp peak at 20,000 daltons when the reaction was run at 12mMMg^{+2} (Thorpe and Ihler, 1974). It is possible but not proven that this is the product of gene G since it migrates very close to a ϕX174 coat protein.

SUMMARY

The use of DNA as messengers directly with procaryotic ribosomes allows the accurate mapping of procaryotic DNA. It seems likely that one of the major uses for this technique will be to map vectors and cloned DNA. It is not yet known whether the technique will yield accurate data when eucaryotic binding sites are studied using either eucaryotic or procaryotic ribosomes, but this is presently under investigation. It seems likely that the system will prove useful for investigating translational control and there is some reason to believe that accurate synthesis of polypeptides directly from single-stranded DNA may be possible.

COMMENTS

Fralick: In polycistronic transcription units in lambda, do you find any differences in binding affinities among structural genes?

Ihler: Not to any significant extent in P_L, P_R and the immunity region.

Nairn: Have you examined polar mutants for any relative differences in ribosome binding affinities among structural genes in a polycistron?

Ihler: No.

Womack: Are ribosome binding affinities different for the structural gene and the homologous messenger RNA?

Ihler: No direct comparison has yet been made. Competitive experiments using purified species of mRNA could be readily done, however.

Garrard: What is known about the sequences of these binding sites? How long are they, and in particular, do any of these sites contain restriction endonuclease sequences?

Ihler: In lambda, C_I and *cro* have been sequenced. Many other sequences are available, in both procaryotic and eucaryotic systems. It is not entirely clear where the binding site sequence *per se* starts and stops since more bases are protected than are needed for binding.

Zink: Is binding strand-specific?

Ihler: Yes, as has been shown in lambda, T7, T4 and Tn10.

Lee: Have the electron microscopy studies been done with salt-washed ribosomes as well as in the presence of initiation factors?

Ihler: No. Salt-washed ribosomes do not bind to any significant extent and the sites used for the residual level of binding have not been examined.

O'Donovan: Were you surprised to find that the specificity of ribosome binding was affected to a much greater extent by the proper initiation factors than by the origin of the ribosomes *per se*?

Ihler: We anticipated that there might be some change in the initiation factor fraction after T4-infection. However, the present understanding of translational control in the context used here is so confused at present that no reasonable prediction can be made.

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LOCALIZATION OF THE BAND II-48C AS THE GENE FOR TYROSINE AMINOTRANSFERASE AND ITS ACTIVATION BY VITAMIN B_6 IN *DROSOPHILA HYDEI*

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ABSTRACT

Incubation of isolated salivary glands in pyridoxine-HCl (Vitamin B_6) at physiological concentrations (10^{-7}M or greater) induces a single puff at the region II-48C. In the presence of Vitamin B_6 (10^{-6}M) a single change in the ^{35}S-methionine incorporation pattern of autoradiograms of SDS polyacrylamide gel was detected. This newly synthesized polypeptide has a molecular weight of approximately 24,000.

The appearance of this new polypeptide is α-amanitin sensitive and thus requires new mRNA synthesis. The synthesis of this polypeptide is correlated with an α-amanitin sensitive increase of tyrosine transaminase activity. SDS-polyacrylamide gel electrophoresis of the band of material showing tyrosine aminotransferase activity results in a polypeptide subunit size of 24,000. Thus an increase of free coenzyme (pyridoxine HCl) within physiological concentrations specifically induces the synthesis of tyrosine transaminase which is in turn dependent on new mRNA synthesis.

INTRODUCTION

The regulation of gene activity is most probably the most important unsolved problem in eukaryotic cell and developmental biology. Because of the difficulty of making direct observations of chromosomes in interphase cells it has been necessary in most cases to study gene activation by indirect methods. There are however a few systems in which it is possible to observe the functioning of localized parts of the genome. The polytene chromosomes of dipteran cells are such an example. Extensive work has been done in mapping *Drosophila* chromosomes and the gross structure of the individual chromosome is well known. Chromosomal puffing, considered to be a reliable indicator of intense gene activity, has been extensively examined in dipteran cells. Thus, it has been possible to examine the temporal sequence of puffing (Beerman, 1952) of RNA synthesis (Kyel, 1964) and to relate these events to stages in the differentiation of the larva (Beerman, 1961). More recent work has demonstrated that individual genes may be localized *in situ* hybridization at specific bands on these chromosomes (Pardue, *et al.*, 1970; Wimber and Steffensen, 1970; and Grigliatti, *et al.*, 1973) and that the RNA products of specific puffs can be isolated and characterized (Daneholt and Hosick, 1973).

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We have chosen to examine a system in which the chromosomal site of activation and the molecular species involved in the general process of activation can be determined. We have examined the activation of the locus II-48C in the *Drosophila hydei* salivary polytene chromosomes.

It has been shown that heat shock (incubation at 37 C), recovery from an anaerobic state or conditions where terminal respiration was inhibited by rotenone, amytol, etc. (van Bruegel, 1966; Leenders and Berendes, 1972) induces puffing at 6 chromosomal loci in *D. hydei* salivary polytene chromosomes. In all of these treatments II-48C was one of the puffs induced. To examine this result further a study was made of the utilization of citric acid cycle intermediates and of amino acids as substrates for respiration under conditions in which respiratory activity was enhanced experimentally and region II-48C and the other loci were puffed. Increased utilization was observed of both isocitric acid and the amino acid tyrosine (Leenders and Knopien, 1973). For these reasons Vitamin B_6 (pyridoxine-HCl) which serves as a coenzyme for tyrosine transaminase and other transaminase enzymes was added to the incubation medium. Under these conditions as a puff appeared at II-48C alone (Leenders, *et al.*, 1973).

We have recently demonstrated that there is an α-amanitin sensitive increase in tyrosine aminotransferase activity which occurs in these cells whenever the puff (II-48C) is formed (Brady and Belew, *In Press*). We report here the kinetics and specificity of puff induction in response to Vitamin B_6 and several of its analogs, the change in protein synthesis resulting from puff induction and the correlation of the molecular weight of newly synthesized polypeptide with that of tyrosine aminotransferase.

MATERIALS AND METHODS

Puff Induction

Salivary glands were hand isolated in Ringer solution from late third instar larvae of *D. hydei* (Leenders and Berendes, 1972). Isolated glands were transferred to Poels Medium (Poels, 1972) pH 6.9 at 24 C and incubated in the dark. Appropriate concentrations of Vitamin B_6 (pyridoxine HCl), pyridoxal-5'-phosphate, pyridoxal, pyridoxalamine phosphate, and pyridoxalamine were added to the above medium, then adjusted to pH 6.9 to examine their ability to induce puffs. All incubations were carried out on a well slide containing 10 μl of medium in a moist chamber. In each case 5 intact pairs of glands were used. At the end of the incubation glands were rinsed in Ringer solution, fixed in 3:1 (ethanol:acetic acid) for 15 min and then stored in a freezer (-20 C) in 70% ethanol. Glands were stained in acetic-orcein and squashed in 45% acetic acid. The diameter of puff II-48C relative to that of II-47B was measured. A minimum of 25 measurements were made for each point, from at least 5 different pairs of glands.

Inhibition of RNA Synthesis

In those experiments where mRNA synthesis was to be inhibited α-amanitin (100 μg/ml, Calbiochem) was added to the incubation medium.

Protein Synthesis

Five pairs of salivary glands were incubated in 10 μl Poels medium or Poels medium containing the appropriate concentration of Vitamin B_6 for 90 min at 24 C and then transferred to a 10 μl drop of culture medium containing 10 μCi ^{35}S-methionine for 15 min (S.A. 600 Ci/mmole Amersham).

The glands were rinsed in Ringer solution containing a 100-fold excess of nonradioactive methionine and then rinsed with several drops of 10% TCA (5 C). The glands were then transferred to 1 ml conical centrifuge tubes containing cold 10% TCA followed by 95% ethanol, and chloroform-methanol (1:1) for 15 min each; and dried at 37 C for at least 30 min. The dried glands were dissolved in 50 μl of sample buffer (Lewis, *et al.*, 1975) and heated in boiling water for 10 min. Samples were loaded onto 10% acrylamide-SDS gels (Lewis, *et al.*, 1975) and run at 8 mA/gel. Gels were stained with Coomassie Blue B, destained, sliced longitudinally into 1 mm slices and dried. Autoradiography was performed using Kodak No Screen X-ray film. Exposure time was approximately 12 hrs.

Enzyme (TAT) Gels

Glands were incubated as described above. Five pairs of glands were ground in pH 8.3 Tris-glycine buffer (Davis, 1964). Electrophoresis was carried out on 7% gels (Davis, 1964). 10^{-3} M α-ketoglutarate and 10^{-2} M pyridoxine HCl were added to the upper buffer. Gels were run at 2mA/tube for 10 min and then at 4mA for the remainder of the run. Buffer temperature was maintained at 5 C. Gels were stained for tyrosine aminotransferase (TAT) activity (Shaw and Prasad, 1970).

TAT Subunits

Non-denaturing gels were run as described in the preceding section and frozen at -20 C immediately after electrophoresis. Once the location of the TAT band had been determined from other gels of the same run, the frozen gels were sliced into 1 mm sections and the single section containing the TAT activity was removed and placed in 30 μl Tris-SDS buffer (Lewis, *et al.*, 1975). The solution was then placed in boiling water for 10 min. Samples of the extracted proteins were placed on SDS-acrylamide gels (Mac Gillivary, *et al.*, 1972) as described above.

Molecular Weight Determinations

Molecular weights were determined by the method of Weber and Osborn (1969). The following molecular weight marker proteins were employed. Cytochrome C (12,400 Daltons), Myoglobin (17,500 D), Chymotrypsinogen (24,000 D),

Ovalbumin (45,000 D) and Bovine serum albumin (68,000 D). The relative mobility of each protein in the gel was calculated and plotted against the log of the molecular weight to construct a standard molecular weight curve.

RESULTS

Kinetics of Puff Induction

The relative size of the region II-48C was compared with region II-47B, an easily identifiable band on the second chromosome not involved in the puff (Fig. 1; for all points n = 20; standard error is shown). solated glands were incubated in 1 x 10^{-2} M Vitamin B_6. This concentration was shown to produce maximal puff induction. From the graph it can be observed that puffing begins immediately and that the puff size is greater than the control after 5 min. Puff diameter at the end of a 2 hr incubation is approximately twice that of the controls (Fig. 2).

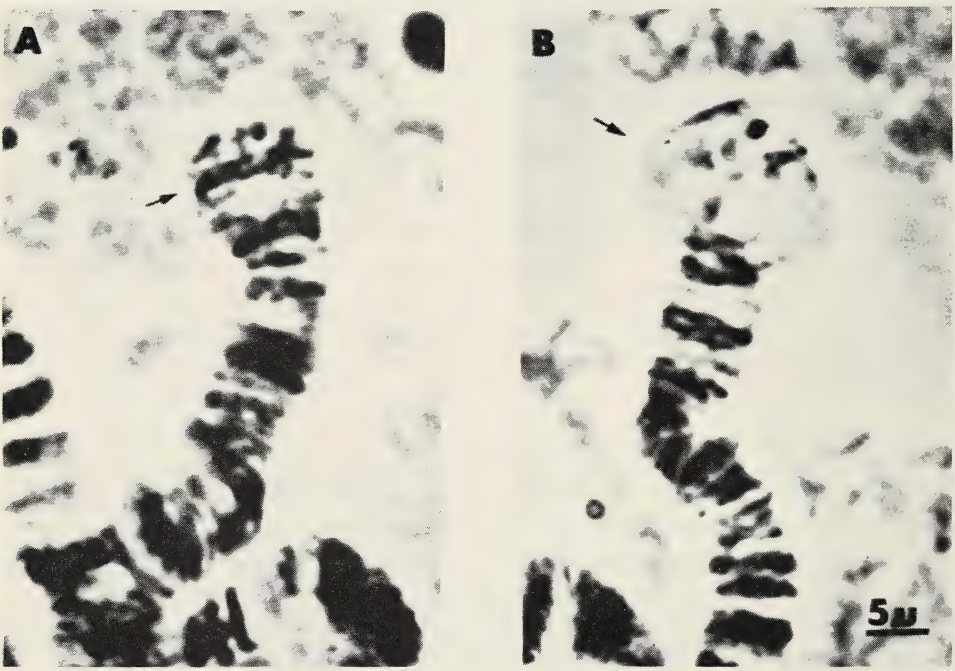

Figure 1. Phase micrographs of orcien stained *D. hydei* salivary polytene chromosome showing terminal portion of chromosome II. Both pictures are of chromosomes from the same pair of salivary glands. In A the gland was incubated for 2 hrs in Poels medium minus Vitamin B_6. In B the gland was incubated for 2 hrs in medium containing 1.5 x 10^{-2} M Vitamin B_6. Arrows indicate the position of band II-48C.

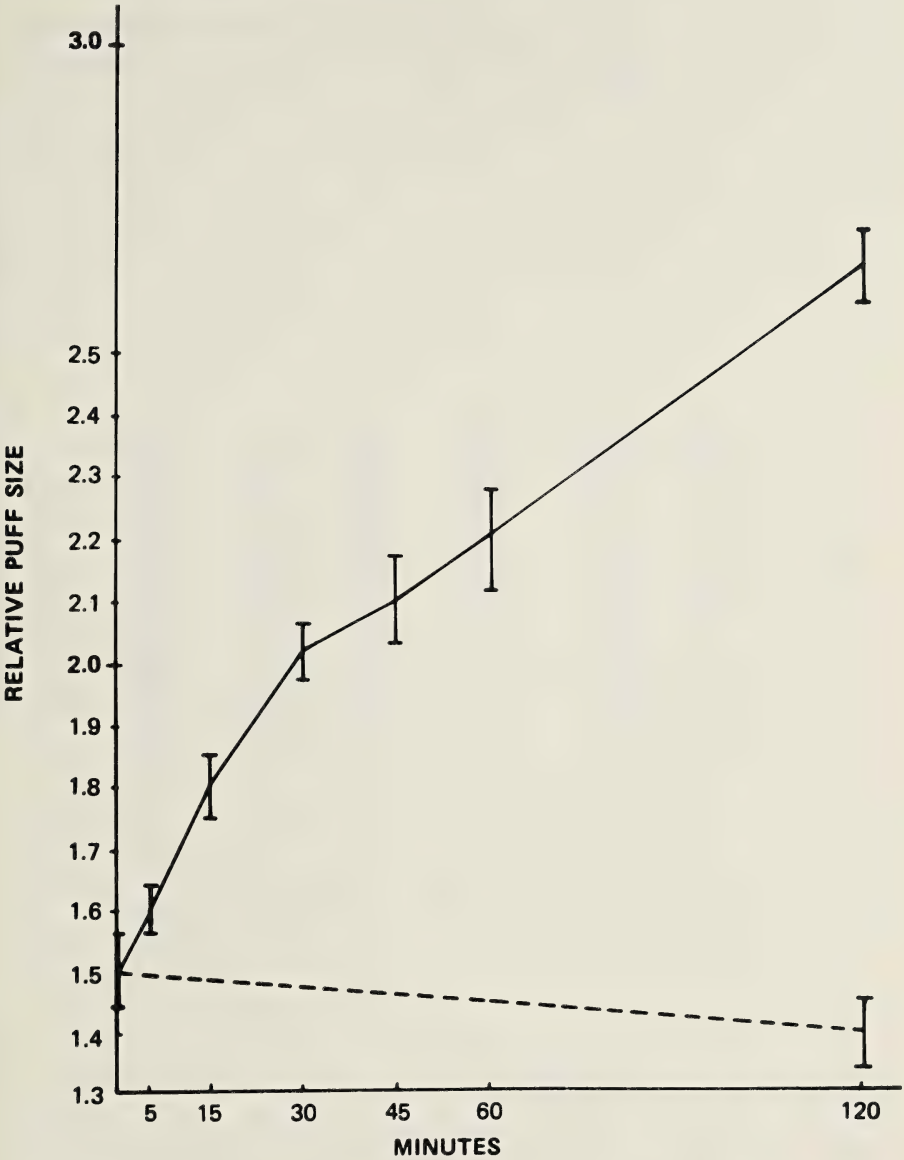

Figure 2. Kinetics of puff II-48C induction by 1.5 x 10^{-2} M Vitamin B_6 (pyridoxine HCl). Puff measurements were made relative to band II-47B.

Response to Various B_6 Concentrations

Although maximal response is obtained with 10^{-2} M, this concentration is exceedingly high and nonphysiological. Concentrations higher than 10^{-2} M are almost immediately lethal to isolated glands. Concentrations from 10^{-7} to 10^{-2} M

were used and even at the lowest concentration a significant increase in puff diameter above that of the control could be observed. There was a linear relationship of puff diameter with the concentration of Vitamin B_6 from 10^{-7} to 10^{-3} M with a logarithmic increase from 10^{-3} to 10^{-2} (Fig. 3). It is at this very high concentration of B_6 that a massive accumulation of ribonucleo protein (RNP) particles is observed in the puff.

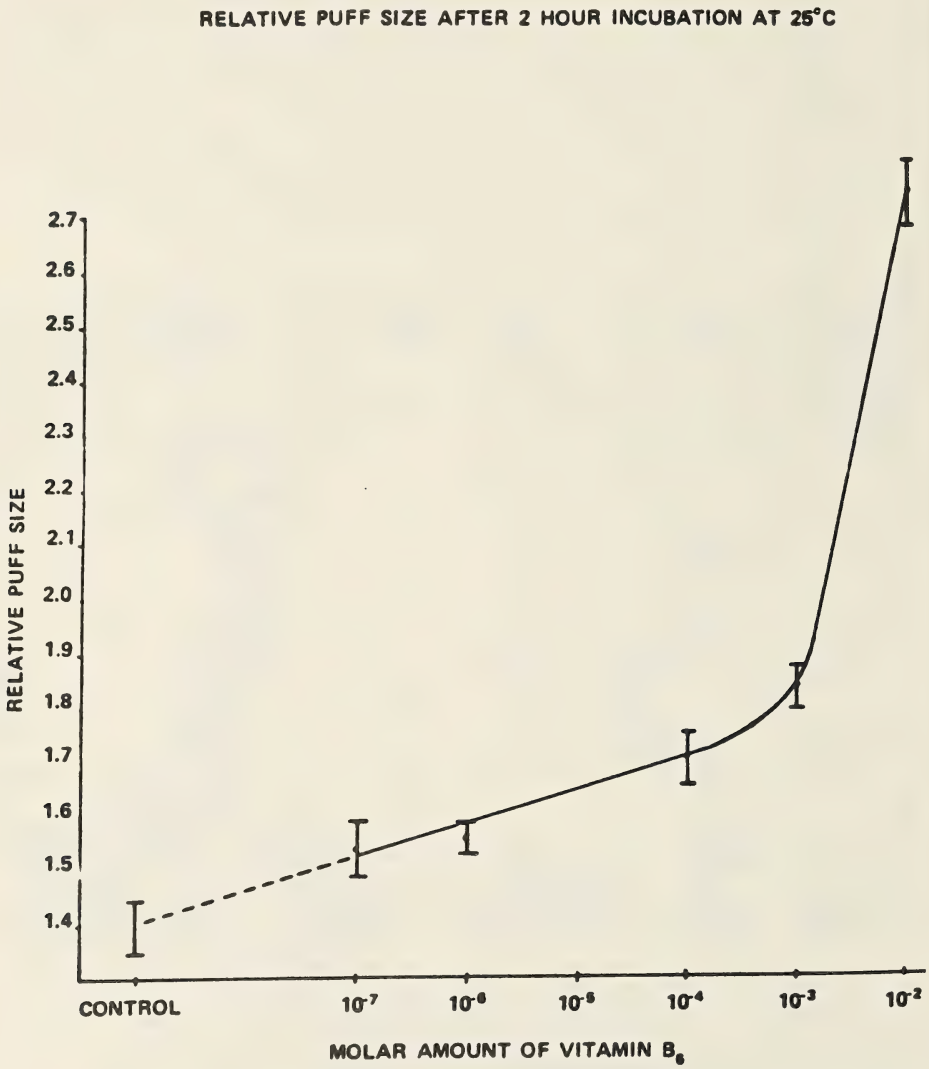

Figure 3. Size of puff II–48C relative to band II–47B after a 2 hr incubation in varying concentrations of Vitamin B_6.

Changes in Protein Synthesis as a Result of Puff Induction

Changes in the pattern of protein synthesis were observed in glands incubated in Vitamin B_6 at concentrations from 10^{-6} to 10^{-3} M. After 2 hrs incubation in B_6, the last 15 min of which included ^{35}S-methionine, proteins were extracted and run on SDS-urea gels. The gels were stained, sliced and autoradiograms of the gels prepared (Fig. 4). Controls indicating the pattern of protein synthesis at the beginning of the experiment and also glands incubated in the absence of B_6 were run. In all cases a single new polypeptide of molecular weight 24,000 D could be observed in glands incubated in B_6 and not in the controls. Glands incubated in B_6 plus α-amanatin to block new mRNA synthesis also lacked the band of newly synthesized protein at 24,000 D.

Figure 4. Autoradiograms of SDS-polyacrylamide gels. The protein was extracted from salivary glands which had been incubated under the following conditions: (a) 15 min in Poels medium with ^{35}S-methionine. (b) 10^{-6} M B_6 in Poels for 2 hrs. Last 15 min in ^{35}S-methionine. (c) 10^{-5}M B_6 in Poels medium for 2 hrs. Last 15 min in ^{35}S-methionine.(d) 10^{-4} M B_6 in Poels medium for 2 hrs. Last 15 min in ^{35}S-methionine. (e) 10^{-3} M B_6 in Poels medium for 2 hrs. Last 15 min in ^{35}S-methionine (f) 2 hrs in Poels medium without B_6. Last 15 min in ^{35}S-methionine. Arrows indicate B^6 induced protein.

Identification of the Newly Synthesized Polypeptide as the Subunit TAT

Non-denaturing gels (7% acrylamide) were prepared according to the method of Davis (1964). Total cell extracts from either salivary glands or entire larvae either induced with B_6 or heat shock or uninduced controls were run on these gels and the gels were stained for TAT (Fig. 5). In all cases a single band of stained material was observed on the gels. This indicated that the uninduced and induced forms of the enzyme were of the same identity. A control omitting tyrosine as the substrate in the stain reaction was done for each set of gels. In these gels no band of stained material could be observed, indicating the specificity of this enzyme for tyrosine.

The 1 mm slice of a gel containing the TAT activity was removed and the proteins in the gel extracted in a denaturing buffer and run on SDS gels. On these

Figure 5. Nondenaturing gels of protein from *D. hydei* salivary glands which had been incubated in 10^{-6} M Vitamin B_6 for 2 hrs. (a) Stained without tyrosine as substrate in the staining reaction. (b) Stained with tyrosine as stain reaction substrate. Arrow indicates the single tyrosine aminotransferase band.

Figure 6. SDS-polyacrylamide disc gel of the band of tyrosine aminotransferase activity extracted from nondenaturing gels. The arrows note the 4 bands on the protein stained gel corresponding to molecular wts of 24,000, 48,000, 72,000 and 96,000 Daltons.

gels 4 bands could be observed (Fig. 6). These had molecular weights of 24,000, 48,000, 72,000, and 96,000 D respectively. We have interpreted these results to indicate that we are dealing with primarily 1 polypeptide in the TAT enzyme stained portion of the gel, and that this polypeptide has a molecular weight of 24,000 D, and that under the conditions in which these gels were run the enzyme was not totally denatured. The molecular weight of 24,000 D is the same as that for the newly synthesized polypeptide observed after B_6 treatment of these glands.

CONCLUSIONS

The induction of intense gene activity (puffing) in the region II-48C of *D. hydei* salivary polytene chromosomes is brought about by Vitamin B_6 and those analogues which are able to function in this system as coenzymes for TAT (Brady and Belew, *In Press*). Those analogs not serving as coenzymes are also unable to elicit puff induction. Autoradiograms indicate a migration and accumulation of ^3H-B_6 in the nucleus (Belew and Brady, *In Press*). The inability to induce a puff in this region in isolated nuclei even in the presence of B_6 suggests that a cytoplasmic factor in addition to B_6 is necessary to induce puffing. The puffing response is correlated with an increase in TAT activity in these cells (Brady and Belew, *In Press*) as well as with the synthesis of a single new polypeptide with a molecular weight of 24,000 D. This molecular weight corresponds with the subunit size of TAT as determined with SDS acrylamide gels.

The results to this point indicate that we are dealing with a eucaryotic system which has a positive coenzyme feedback on the genome. These results are consistent with the hypothesis that when there is an excessive amount of B_6 in these cells (10^{-7} M or greater) this compound (or several of its analogues) binds to a factor in the cytoplasm and migrates to the nucleus where it is directly involved in the activation of the region II-48C.

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COMMENTS

Garrard: Does puff induction require protein synthesis?

Brady: Our preliminary work incubating isolated salivary glands in concentrations of Vitamin B_6 which normally induce a large puff but also in the presence of protein synthesis inhibitors (cyclohexamide at 15 μg/ml) indicates that protein synthesis is not required for the induction of this particular puff.

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CHANGES IN NONHISTONE CHROMOSOMAL PROTEINS CORRELATED WITH INDUCTION OF PUFF II-48C IN *DROSOPHILA HYDEI* POLYTENE SALIVARY GLAND NUCLEI

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ABSTRACT

Salivary glands from early third instar larvae were mass isolated and incubated at 24 C in Poels culture medium or in Poels medium with 1.5 x 10^{-2}M Vitamin B_6 (pyridoxine HCl). The latter treatment induces the formation of a large puff at region II-48C on the salivary polytene chromosomes. After incubation the polytene nuclei were isolated. The nonhistone chromosomal proteins (NHCP) were extracted and electrophoresed in SDS-polyacrylamide disc gels. A 46,000 D and a 40,000 D protein band were found in glands incubated in Vitamin B_6 but not in the control glands. To determine whether these proteins were specific to the induction of puff II-48C, the changes in NHCP were followed when the ecdysone puffs were induced. A 40,000 D protein was found in glands incubated in ecdysone (1 mg/ml) but not in control glands. Ecdysone puffs occur naturally in polytene chromosomes of late third instar larvae. NHCP extracted from these larvae also contain a 40,000 D protein but not the 46,000 D protein. The appearance of a 40,000 D protein was found whenever puffs were induced, while the 46,000 D protein appears to be specific to the induction of puff II-48C.

INTRODUCTION

The polytene chromosomes of *Drosophila hydei* salivary glands offer a unique eucaryotic system for the examination of gene regulation. These are interphase chromosomes and they are composed of numerous longitudinally running chromatin fibers. These fibers are coiled at specific intervals to form darkly staining bands and are extended in the interband region (Brady, *et al.*, 1977).

Both during normal development and as a result of experimental manipulation, puffs (morphological evidence of intense gene activity) occur in individual bands of the chromosomes (Beerman, 1952; Ritossa, 1964; Berendes, *et al.*, 1965; Leenders and Beckers, 1972; Leenders and Berendes, 1972).

A single large puff at region II-48C on the salivary gland chromosomes of *D. hydei* is induced *in vitro* by incubation of isolated salivary glands in 1.5 x 10^{-2}M Vitamin B_6 (pyridoxine HCl) (Leenders, *et al.*, 1973). One of the first events in puff formation is increased acidic protein specific dye binding at the puff site (Berendes, 1968; Holt, 1970, 1971). This accumulation occurs in the absence of new protein synthesis (Holt, 1971; Goodman, *et al.*, 1976).

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The nonhistone chromosomal proteins (NHCP) are acidic and extremely heterogeneous (Elgin, *et al.*, 1973). Both quantitative and qualitative variability in these proteins occurs among different tissues within 1 organism and, at different developmental stages, within the same tissue (Elgin, *et al.*, 1973; Gilmour and Paul, 1971; MacGillivary, *et al.*, 1972). The majority of these acidic proteins have enzymatic or structural functions common to all chromatin (Swift, 1964). However, Gilmour and Paul (1970), Kleinsmith, *et al.*, (1970), and Kostraba and Wang (1973) demonstrated that some NHCP may function in gene activation.

Goodman, *et al.*, (1976) suggested that the accumulation of acidic proteins at the puff site in response to ecdysterone is due to an influx of cytoplasmic proteins. Helmsing and Berendes (1971) and Helmsing (1972) demonstrated the accumulation of a 41 kilo Dalton (kD) protein in response to ecdysterone treatment and a 23 kD protein in response to heat shock or anaerobic shock in *D. hydei* salivary gland nuclei. Both of these latter treatments induce the same series of puffs. Puff II-48C, which is selectively induced by Vitamin B_6 (pyridoxine HCl), is 1 of the 6 puffs resulting from heat shock or anaerobosis. Since Helmsing and Berendes (1971) found a change in the nonhistone chromosomal protein pattern after induction of all 6 puffs, this work was undertaken to determine what changes (if any) in the nonhistone chromosomal proteins would be found when only puff II-48C was induced to form.

MATERIALS AND METHODS

Mass Isolation of Salivary

Gram quantities of salivary glands were isolated using a modification of the technique of Boyd, *et al.*, (1968). Mass cultures of *D. hydei* were raised under standard conditions at 24 C. Larvae were synchronous in growth, coming from eggs layed over a 2 hr period. Sixty grams of third instar larvae (either 136 hrs or 184 hrs old) were used in each experiment. Each experiment reported was performed at least 3 times. The larvae were spread on a siliconized glass plate and oriented with a strong light. The internal organs were extruded by rolling over the larvae from the rear with a steel rod. Tissues were washed from the rod and glass plate with cold (4 C) Ringers solution (Boyd, *et al.*, 1968). All subsequent operations were carried out at 4 C. The larvae were allowed to settle for 5 min. The fat bodies were poured off the top and the remaining suspension was then swirled and poured through a 1.1 mm mesh sieve to remove larval carcasses. The settling and sieving procedures were repeated 4 times. The suspension was poured through an 0.85 mm mesh to remove large pieces of gut. The suspension was then swirled and allowed to settle for 30 sec. Salivary glands preferentially adhere to the walls of the original beaker and were washed from the walls into a small beaker where contaminating tissues were removed with a Pasteur pipette. This procedure was repeated 6 times and the salivary glands pooled.

The salivary glands were pelleted at 60 x g for 5 min in a refrigerated centrifuge and the supernatant was poured off.

Incubation of the Glands

A variety of incubation conditions were employed to induce the different series of puffs. In each case the isolated glands were divided into 2 equal portions. One of the portions was suspended in Poels medium (Poels, 1972) and incubated at 24 C for either 30 or 90 min. This portion served as the control for each experiment. The other portion was treated to induce the specific puffs desired.

To induce the heat shock puffs the preparation was incubated in Poels medium at 37 C for 30 min.

To induce puff II-48C the glands were incubated in 1.5×10^{-2} M Vitamin B_6 or 1.5×10^{-2} M pyridoxal 5' phosphate at 24 C for 30 min.

To induce ecdysone puffs the glands were incubated in Poels medium with 1 mg/ml ecdysone for 90 min at 24 C.

At the end of the incubation period the incubation medium was pipetted off and discarded.

Isolation of Nuclei

Nuclei of the salivary glands were isolated and purified according to the procedure of Elgin and Boyd (1975). The glands were transferred to a homogenizer and broken in 3 ml of nuclear isolation buffer (0.11 M NaCl, 0.002 M KCl, 0.0025 M $MgCl_2$ and 0.1 M Tris-HCl at pH 7.2) which contained 0.001% spermidine and 0.2% Triton-X. Fragments were allowed to settle and the supernatant was removed. The homogenization was repeated with fresh buffer. The supernatants were combined and brought to 0.5% Triton-X. The nuclear suspension was filtered through a 53 μm mesh and washed with 100 ml nuclear isolation buffer. The collected material was washed and centrifuged at 40 x g for 5 min to pellet the nuclei. Nuclei were resuspended in 10 ml of nuclear isolation buffer containing 1.67 M sucrose and layered on 2 ml buffer with 2.3 M sucrose in a polyallomer tube. The tubes were centrifuged at 40,000 rpm for 42 min in a Beckman 42 preparative ultracentrifuge to form a nuclear pellet.

Nonhistone Chromosomal Protein Extraction

The nonhistone chromosomal proteins were extracted using the method of Konings, *et al.*, (1970). The nuclear pellet was suspended in 1 ml of Ringers solution (Leenders and Berendes, 1972) with 0.16 mM EDTA and 1% SDS. Three ml of phenol saturated with 0.25 M Tris-HCl (pH 7.8) were added and the suspension vortexed for 10 min at 16,000 x g. The phenol layer was removed and reserved. The phenol extraction was repeated for the aqueous and interphase layers. Both phenol layers were then combined with 2 volumes of ice cold acetone containing 0.1 M acetic acid. The solution was kept at 0 C for at least 2 hrs. The precipitate that formed was pelleted and washed with 1:1 ethanol:ether, then with ether and dried.

SDS Polyacrylamide Gel Electrophoresis

The dried precipitate was mixed with 20 μl of 0.01 M sodium phosphate buffer (pH 7.6) containing 0.1% SDS, 0.14 M B mercaptoethanol and 4 M Urea and incubated for 2 hrs at 37 C. The protein was then heated to 100 C for 1.5 min. One drop glycerol was added and the preparation was applied to the top of SDS-acrylamide disc gels (Weber and Osborn, 1969) which had been modified to carry a 3% acrylamide, 0.1% SDS, phosphate buffered stacking gel. The gels were run at 8mA/gel for 4 hrs and stained in 0.25% Coomassie Brilliant Blue B dissolved in 45.5% methanol and 9% acetic acid. The gels were stained overnight, rinsed in distilled water and electrophoretically destained for 30 min in 7.5% acetic acid and 5% methanol. A Beckman 24 spectrophotometer was used to scan the gels at 550 nm. The scans of the gels were examined for changes in the protein pattern. Gels were stored in 10% acetic acid.

Molecular Weight Determinations

Molecular weights were determined by the method of Weber and Osborn (1969). The following molecular weight marker proteins were employed. Cytochrome C (12.4 kD), Myoglobin (17.5 kD), Chymotrypsinogen (24 kD), Ovalbumin (45 kD) and Bovine serum albumin (68 kD). The relative mobility of each protein in the gel was calculated and plotted against the log of the molecular weight to construct a standard molecular weight curve (Fig. 1).

RESULTS

Induction of Puff II-48C

A number of diverse treatments of isolated salivary glands from *D. hydei* result in the formation of a large puff at band II-48C. *In vitro* incubations in Vitamin B_6 (pyridoxine HCl) or pyridoxal 5' phosphate induce only puff II-48C, whereas heat shock or anaerobic shock results in a series of 6 puffs (II-32A, II-36A, II-48C, III-51B, III-58B and IV-81B) one of which is II-48C. Three treatments were employed to determine what effect, if any, the induction of puff II-48C had on the pattern of nonhistone chromosomal proteins (NHCP) in salivary gland nuclei from late third instar larvae. Figures 2 and 3 show densitometric scans of NHCP prepared from nuclei with no induced puffs (controls) and from nuclei with puffs induced by 1 of the following treatments: 1.5×10^{-2} M Vitamin B_6, 1.5×10^{-2} M pyridoxal 5' phosphate or heat shock at 37 C. It should be noted that all of the treatments result in the appearance of a 46 kD protein after 30 min incubations. This protein is never observed in control glands incubated in Poels medium (Poels, 1972) at 25 C.

The apparent molecular weights of the proteins were determined by referring to the standard molecular weight curve (Fig. 1). This standard curve plots the relative mobility of a protein in the gel against the log of the molecular weight. The relative mobility of each protein was determined by the method of Weber and Osborn (1969).

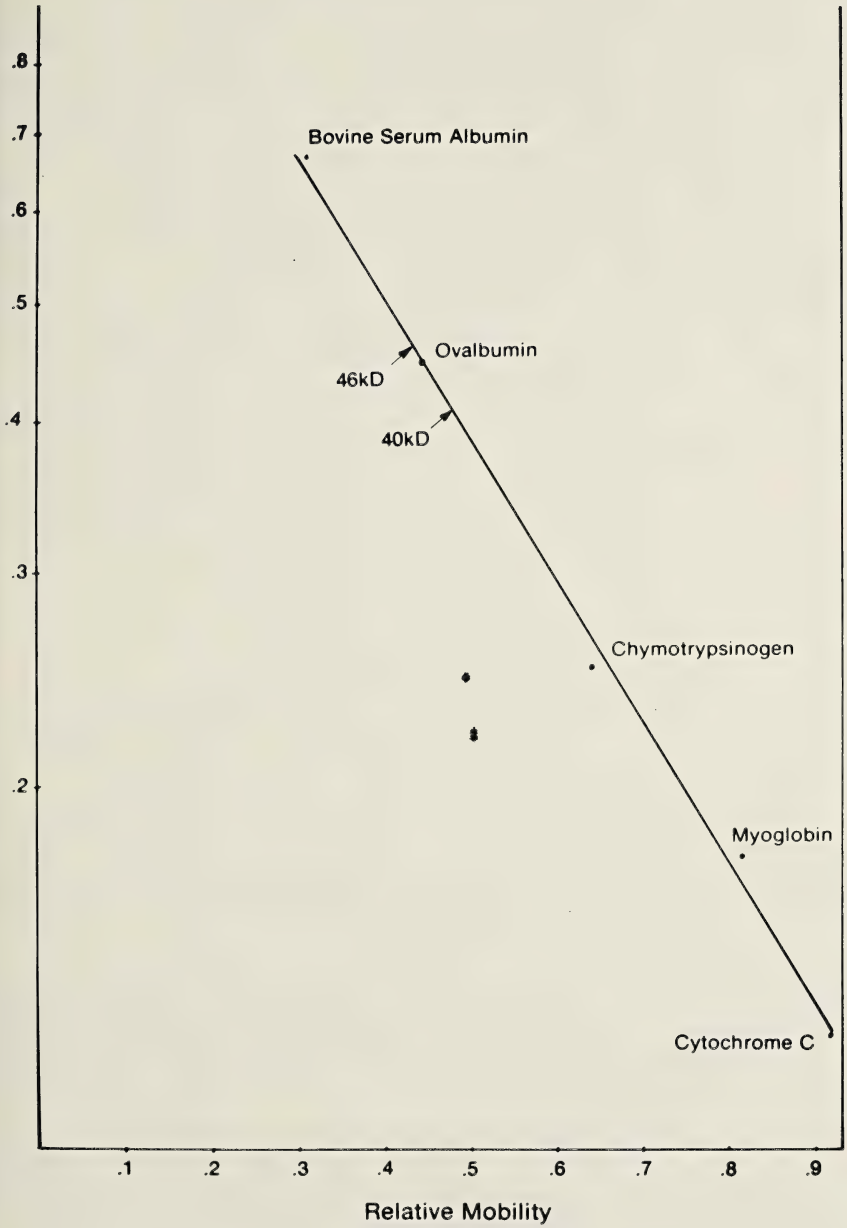

Figure 1. Standard molecular weight graph. The calculated relative mobility is plotted against the log of the molecular weight for each known molecular marker protein. The arrows show the relative mobility of the 46 kD and the 40 kD proteins found in the nonhistone chromosomal protein preparations.

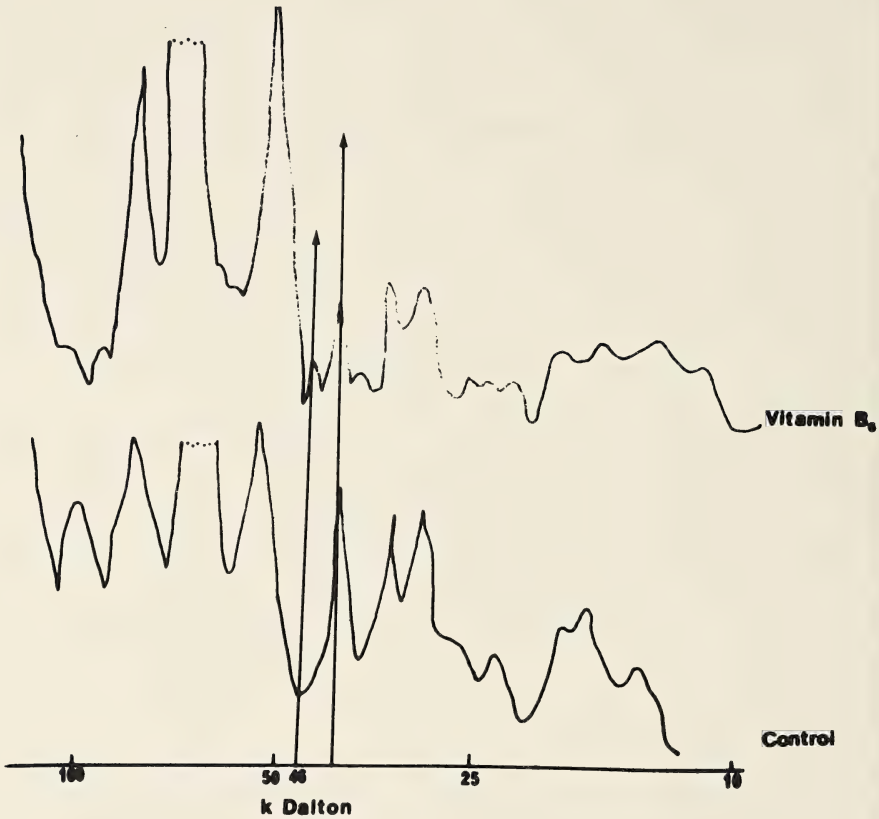

Figure 2. Densitometric scans of SDS polyacrylamide gels are compared. The NHCP were isolated from late third instar salivary gland controls and from those incubated for 30 min in 1.5 x 10^{-2}M Vitamin B_6.

The polytene nuclei from late third instar larvae have a series of puffs arising as a part of normal development in these insects. This complex series is induced by ecdysone secretions in the larvae and does not include puff II-48C or any of the other heat shock puffs.

To determine whether there were general changes in the NHCP pattern that occurred any time puffs were induced, early third instar larvae (which lack observable salivary gland nuclear puffs) were examined. A comparison of the NHCP found in early and late third instar larvae in which no experimental puffs had been induced reveals the presence of a 40 kD protein in the late third instar larvae which is not found in the early third instar larvae (Fig. 4).

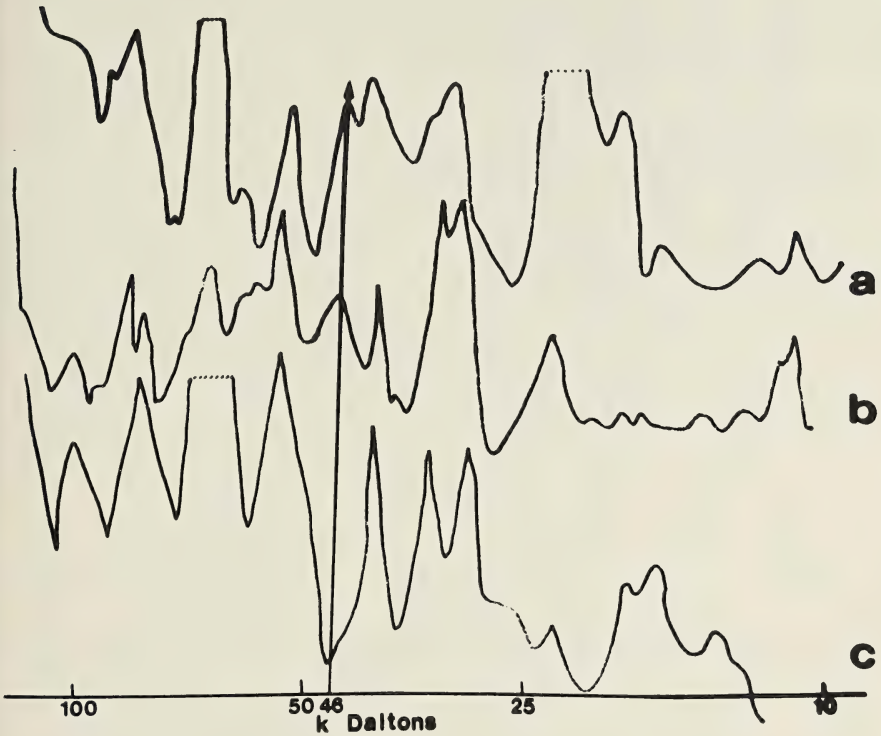

Figure 3. Densitometric scans of NHCP which were run on SDS polyacrylamide gels. The NHCP were extracted from late third instar salivary glands incubated a) in Pyridoxal PO_4, b) at 37 C heat shock or c) at 24 C in Poels medium (control). The arrow indicates the location of the protein band of molecular weight 46 kD.

Figure 5 compares the NHCP from early third instar controls and early third instar larval salivary glands that were incubated in Vitamin B_6. Puff II-48C is induced to form in early third instar salivary gland nuclei by incubation in Vitamin B_6. Both a 40 kD and a 46 kD protein appear in the NHCP from glands incubated in Vitamin B_6.

Ecdysone Induced Puffs

To determine if the 2 proteins which were found to accumulate in the nucleus following incubation in Vitamin B_6 were associated specifically with the induction of puff II-48C (or the other heat shock puffs) or were associated with the general phenomenon of puff induction, ecdysone puffs were induced *in vitro*.

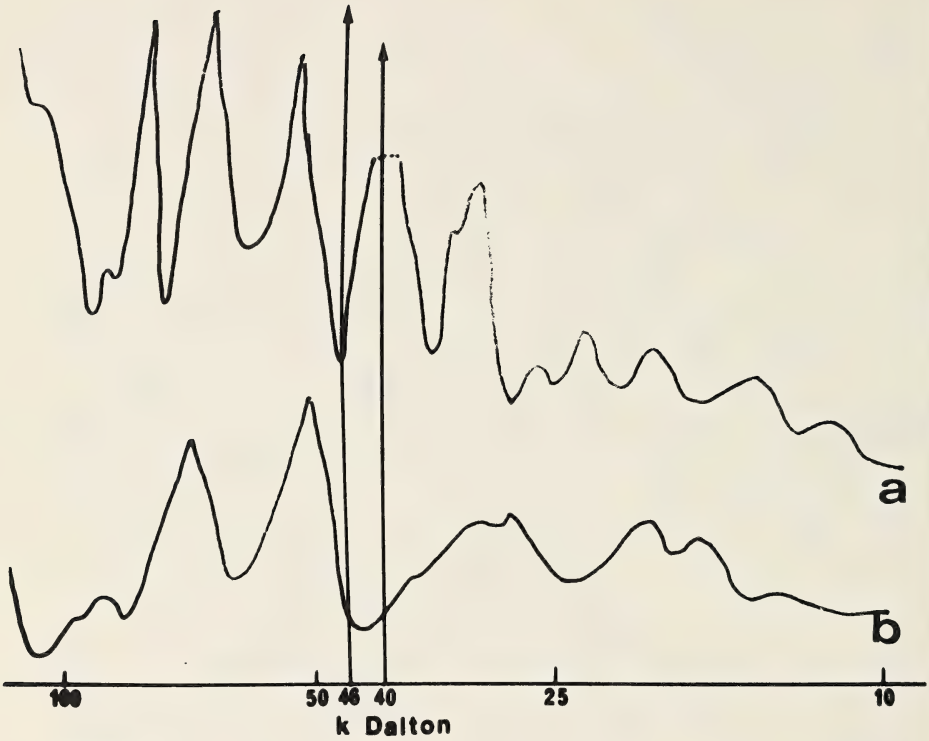

Figure 4.

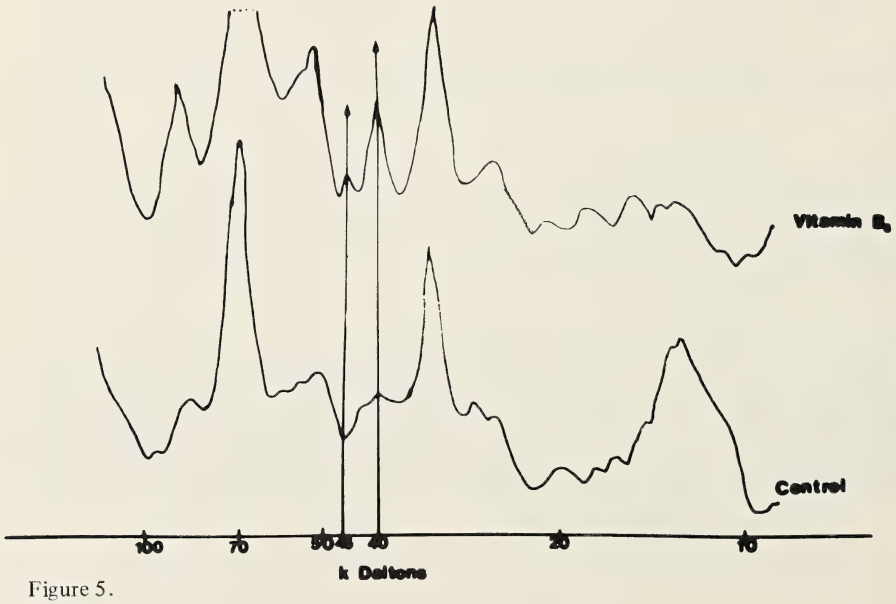

Figure 5.

(Figures 4 and 5 Continued)

Figure 4. A comparison of densitometric scans of NHCP run on SDS polyacrylamide gels from a) late third instar salivary gland controls with those from b) early third instar controls.

Figure 5. Densitometric scans of SDS polyacrylamide gels are compared. The NHCP were isolated from early third instar salivary gland controls and from those incubated in 1.5 x 10^{-2}M Vitamin B_6.

Isolated salivary glands from early third instar larvae were incubated in Poels medium (Poels, 1972) to serve as a control or in Poels medium with 1 mg/ml ecdysone.The incubations were conducted for 90 min. The latter treatment induced the appearance of the ecdysone puffs.

Examination of Fig. 6 reveals the accumulation of a 40 kD protein in the NHCP of the glands incubated in the ecdysone which is not found in the NHCP

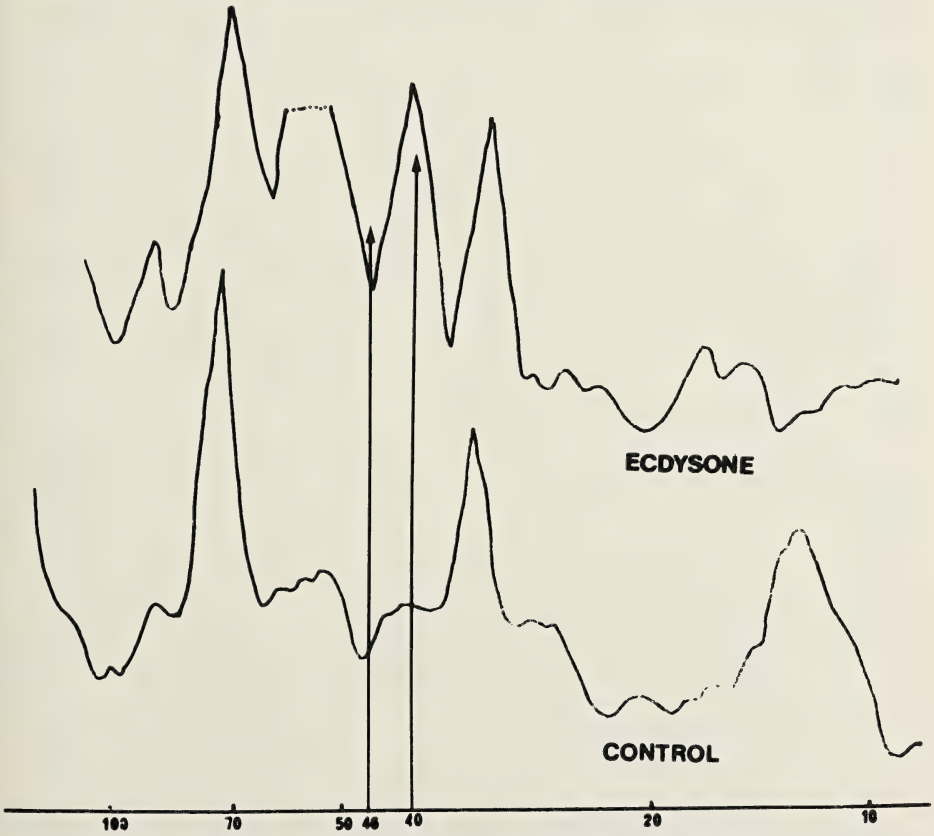

Figure 6. A comparison of densitometric scans of NHCP run on SDS polyacrylamide gels from early third instar salivary gland controls and those incubated in 1 mg/ml ecdysone.

from the control glands. This protein is present in the gel pattern of NHCP from late third instar control larvae preparations (Fig. 7). These late third instar larvae have the normal developmentally induced ecdysone puffs on their salivary gland polytene chromosomes. Thus the same molecular weight protein is found in the nucleus of both glands with naturally occurring and experimentally induced ecdysone puffs, but never in salivary glands that lack any puffs.

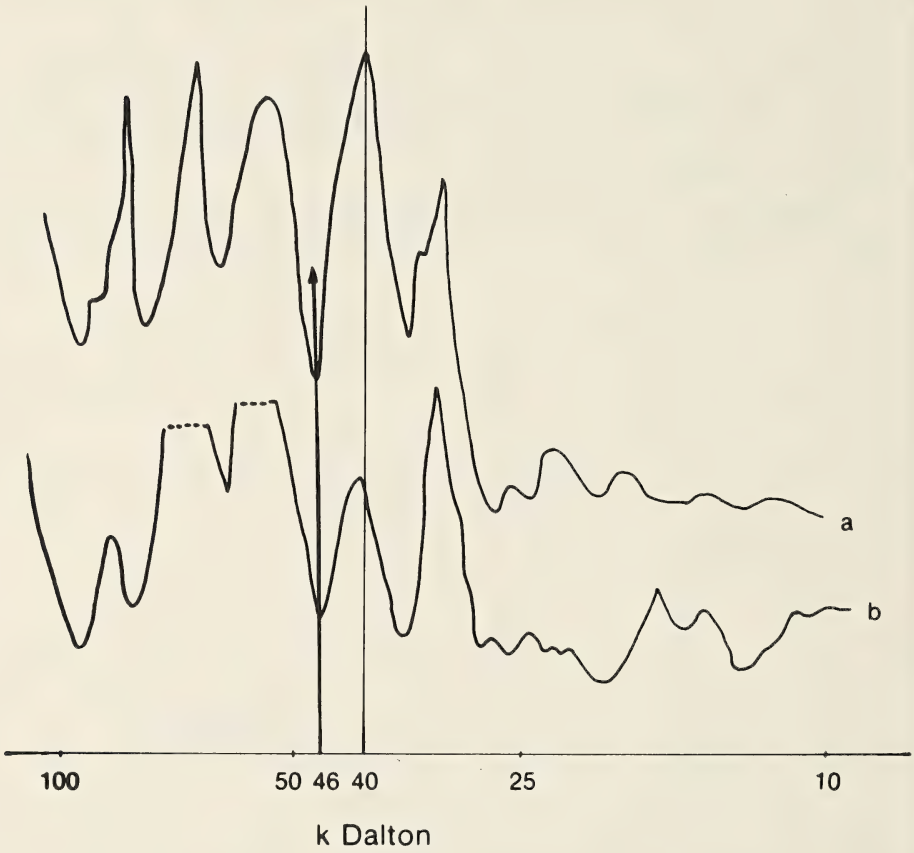

Figure 7. A comparison of densitometric scans of NHCP run on SDS polyacrylamide gels from a) late third instar salivary gland controls and b) those from early third instar salivary glands incubated in 1 mg/ml ecdysone.

DISCUSSION

Acidic proteins, as determined by fast green staining, accumulate in the puffs of *D. hydei* (Holt, 1970, 1971). This staining technique, however, gives no indication of the diversity, number or size of the accumulating proteins. The ability

to induce specific puffs in polytene chromosomes of *Drosophila* salivary gland nuclei has allowed the correlation of acidic protein accumulation at the puff site with the qualitative changes in NHCP.

Incubations of isolated salivary glands in Vitamin B_6 (pyridoxine HCl) or pyridoxal 5' phosphate induce only puff II-48C, whereas heat shock results in a series of 6 puffs (II-32A, II-36A, II-48C, III-51B, III-58B and IV-81B) one of which is II-48C. Our experiments demonstrated the appearance of a 46 kD protein in the nucleus after treatment of salivary glands with Vitamin B_6, heat shock or pyridoxal 5' phosphate, but not after incubation in ecdysone.

This strongly implicates its appearance as being associated with induction of II-48C. The possibility exists that it is associated with the other heat shock puffs, but its involvement in a general puffing phenomenon is eliminated.

Although the data presented here do not allow the determination of the nature of this protein, several possible functions for the acidic proteins associated with the chromatin in eucaryotic cells have been postulated (Swift, 1964). The 4 main possibilities are: transcriptional proteins, structural proteins, packaging proteins, and regulatory proteins.

First, it seems unlikely that the 46 kD protein is part of a transcriptional molecule (e.g. RNA polymerase) since it is not found in nuclei each time puffs are formed. Transcriptional proteins would be necessary for the formation of RNA transcripts from ecdysone puffs as well as from puff II-48C.

Second, the possibility does exist that this protein is a structural protein involved in puff formation. However, it would be necessary to propose that it exhibits some specificity since it is not found in ecdysone puffed nuclei.

Third, puff II-48C, unlike others that have been looked at in this species is known to build up large aggretions of ribonucleoprotein (RNP) particles as the puff forms (Berendes, *et al.*, 1973 and Leenders, *et al.*, 1973). It is possible that this protein is involved in the unique packaging phenomenon associated with this puff. Specific RNP particle proteins with a wide range of molecular weights (Augenlich and Liphin, 1976) have been identified in a number of organisms.

The possibility that these proteins are of a regulatory nature is, of course, very attractive. The evidence points to a specificity of the 46 kD protein since it is not found every time puffs are formed. Puff II-48C fails to form in isolated nuclei incubated in 1.5×10^{-2}M Vitamin B_6, suggesting that cytoplasmic elements are necessary for the induction of this puff (Belew, unpublished results). Further, autoradiography of glands that have been incubated in ^{3}H Vitamin B_6 and squashed, showed localization of label in both cytoplasm and nucleoplasm. Label was found generalized over the chromosomes and directly over region II-48C (Brady and Belew, in prep.). This suggests that Vitamin B_6 by itself or in conjunction with a carrier molecule moves into the nucleus and to many areas of the chromosomes but especially to region II-48C, the future site of the puff. These observations strongly suggest the presence of a carrier molecule for Vitamin B_6 although the nature of this carrier is unknown.

The appearance of the 40 kD protein is noted in each case puffing is found in the nucleus but not in the early third instar larvae which have no observable puffs. This protein is likely to be of a general nature and function, perhaps a protein associated with the general formation of puffs or involved in a RNA packaging function (RNP particle).

The 2 previous reports attempting to correlate changes in NHCP in *D. hydei* in response to puff induction are conflicting. Helmsing and Berendes (1971) observed the accumulation of a 23 kD protein in response to heat shock. Elgin and Boyd (1975) repeated these experiments but were unable to reproduce the results of Helmsing and Berendes (1971). While the gel scans presented by Elgin and Boyd (1975) fail to demonstrate the accumulation of a 23 kD protein, they do contain a peak in the area of 40 kD which is present in heat shock glands but not in controls. This difference was not discussed by the authors. The gels of Helmsing and Berendes (1971) do indeed show a band of protein in gels from heat shocked preparations that is not found in the controls. The gels used by these authors is the same as reported in this paper.

Although an exact comparison of the gels reported here and those of Helmsing and Berendes (1971) is not possible the location of the band in their gel is consistent with the location of the 46 kD band reported here (Fig. 8). It should be noted that these authors neither list the marker proteins used for molecular weight determination nor present the method used for calculating the molecular weight of protein accumulating as a result of puff induction.

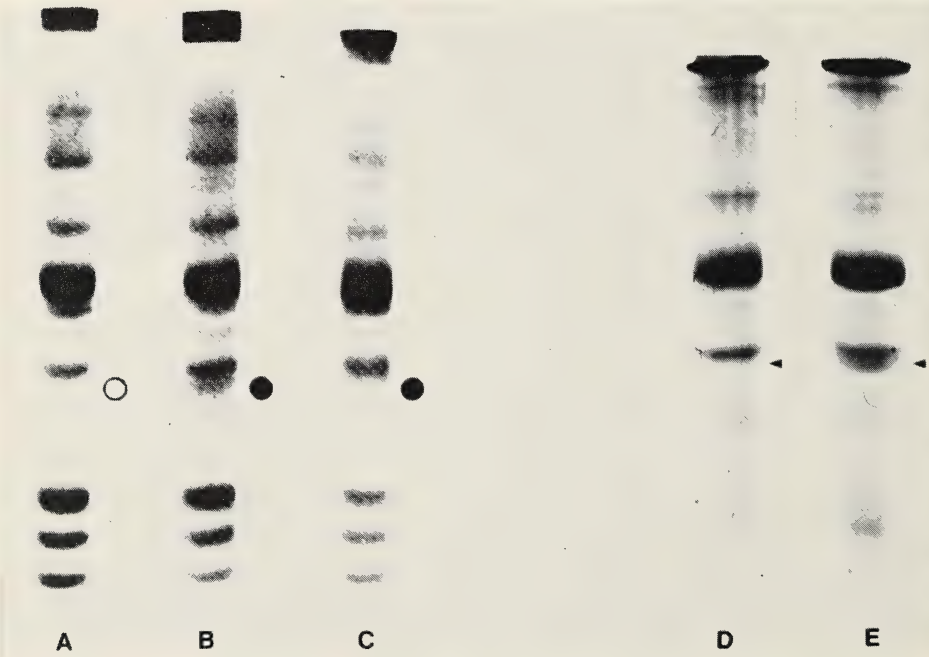

Figure 8.

(Figure 8 Continued).

Figure 8. SDS-polyacrylamide gel separation of phenol soluble nuclear proteins from larval salivary glands. a) Proteins from control nuclei. b) Proteins from larvae which have been subjected to heat shock at 37 C for 30 min prior to isolation of the salivary glands. c) Proteins from larvae which have been subjected to CO_2 treatment 30 min prior to isolation of the salivary glands. d) Proteins from control salivary glands which were incubated in Poels medium (Helmsing and Berendes, 1971). e) Proteins from salivary glands incubated in 1.5 x 10^{-2}M Vitamin B_6 in Poels medium at 24 C (Helmsing and Berendes, 1971). The closed circles indicate the position of the accumulating protein of Helmsing and Berendes (1971). The arrows indicate the position of the protein in the gels reported here. The band is evident in the glands incubated in Vitamin B_6 but not in the control glands.

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PHOSPHORYLATION OF RIBOSOMAL AND RIBOSOME-ASSOCIATED PROTEINS IN DEVELOPING CARDIAC HYPERTROPHY

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ABSTRACT

Rabbit heart muscle has been utilized as a model to investigate the effect of developing cardiac hypertrophy on the phosphorylation of proteins extracted from purified ribosomes isolated from cardiac tissues. Cardiac hypertrophy was induced by the administration of isoproterenol and protein phosphorylation was examined by radioactive labeling in the presence of (^{32}P)-orthophosphate. Protein labeling was carried out by 2 methods: 1) incubation of ^{32}P in a cell suspension of heart muscle cells in Tris-buffered saline and 2) perfusion of the beating heart by the Langendorff technique using a Krebs-Henseleit bicarbonate buffer as perfusate. Labeled proteins were analyzed by electrophoresis on SDS-polyacrylamide gels and liquid scintillation spectrometry of gel slices. Alterations were observed in both the extent of phosphorylation and the number of phosphoproteins isolated from hypertrophic heart tissue as compared to tissue from control animals. Data obtained from the 2 methods were comparable except that the efficiency of labeling was higher by the perfusion method.

INTRODUCTION

Phosphorylation of eukaryotic ribosomal proteins has been shown to occur *in vivo* in a number of systems (Kabat, 1970; Gressner and Wool, 1974; Rankine and Leader, 1975). The phosphate molecules have been shown to be covalently bound to serine or threonine residues of the proteins (Kabat, 1970; Gressner and Wool, 1974; Rankine and Leader, 1975). These phosphoproteins can be grouped into 2 classes: a) proteins that are very tightly associated with ribosomal RNA and cannot be removed without damaging the structure and biological activity of the ribosomes, and b) proteins that are more loosely associated which may be removed by relatively mild conditions such as agitation of a suspension of ribosomes in increasingly high concentrations of salt. Such an isolation procedure yields ribosomes which are relatively intact and retain their biological activity. The former class of proteins is designated as salt-washed ribosomal proteins (hereafter referred to as SWR protein) while the latter class of protein is termed salt-wash protein fraction (SW protein).

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The role of these phosphoproteins requires further elucidation. Attempts to correlate a changing phosphorylation pattern with control of protein synthesis *in vitro* have met with negative results (Eil and Wool, 1973; Kryostek, *et al.*, 1974). It has not been possible to demonstrate that alterations in levels or patterns of protein phosphorylation have affected the activity of the ribosomes in cell-free protein synthesizing systems. However, levels of phosphorylation have been observed to increase in at least 1 small subunit ribosomal protein (S-6) in regenerating rat liver (Gressner and Wool, 1974) and in diabetic rats (Gressner and Wool, 1976). Further, the higher levels of phosphorylation in diabetic rats can be reversed by insulin administration.

We have employed an *in vivo* model system in which the protein synthetic activity can be manipulated thus allowing one to observe any potential accompanying changes in ribosomal protein phosphorylation. The model system is the hypertrophic heart. Compensatory changes to an increased work demand in myocardial tissue are due primarily to hypertrophy, or an increase in the physical dimensions of existing cells, rather than an increase in the number of cells (hyperplasia). This state of accumulation of cellular components presents an opportunity for investigation of changes associated with the metabolic state of the heart. Developing cardiac hypertrophy is accompanied by an increase in total RNA synthesis, in the number of ribosomes, and in the rate of protein synthesis in myocardial tissue (Rabinowitz and Zak, 1972). Accordingly, we have initiated studies on phosphorylation of ribosomal (SWR protein) and ribosome-associated (SW) proteins in cardiac hypertrophy induced by subcutaneous administration of thyroxin or isoproterenol (a β-adrenergic receptor stimulating agent) in rabbits. Phosphorylation has been monitored by following the incorporation of (^{32}P)-orthophosphate into these protein fractions.

MATERIALS AND METHODS

Young male New Zealand rabbits (approx. 3 kg) were treated with thyroxin (2.0 mg/kg in 0.9% saline), isoproterenol (2.5 mg/kg in 0.9% saline), or 0.9% saline for the indicated periods and frequencies. Upon termination of treatment, animals were killed by intravenous injection of sodium pentabarbital. Hearts were immediately excised and processed according to 1 of the 2 labeling techniques outlined in Fig. 1.

Cell Suspension Incubation

The myocardial tissue was prepared according to a modified technique of Glick, *et al.*, (1974). Left and right ventricular slices were removed to phosphate-buffered formaldehyde (10%) for histological studies. The remaining tissue was minced with scissors into pieces approximately 5 mm square and passed through a hand-held tissue press. This material was suspended in a Tris-buffered saline solution (2mM Tris, pH 7.4, 116 mM NaCl, 5 mM KCl) containing 5 mM glucose

and 1 mg/ml of collagenase to a level of 0.6 g tissue/ml incubation medium. The incubation was carried out for 30 min at 37 C in a shaking water bath. Two 0.1 ml samples were removed to separate test tubes and 5 μCi of (^{3}H)-leucine were added to 1 sample. The second sample was frozen to be used for the determination of protein concentration. (^{32}P)-orthophosphate was added to the bulk of the material at a level of 1 mCi/3.4 g tissue. Both (^{3}H)-leucine and (^{32}P)-orthophosphate containing cell suspensions were further incubated for 1.5 hrs, then frozen at -50 C in a dry ice:acetone mixture and stored overnight before ribosome isolation.

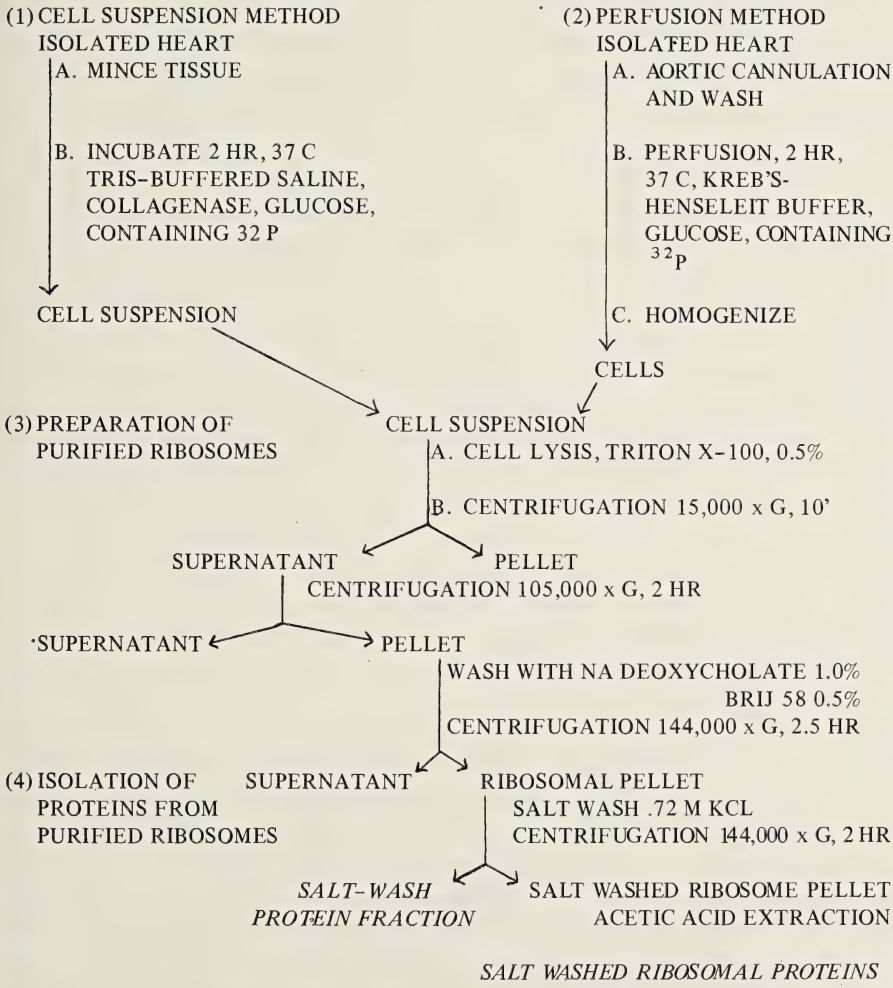

Figure 1. Ribosomal protein isolation procedure.

Perfusion Method

The excised heart was perfused according to the method of Langendorff (1895). This is a reverse flow technique in which the perfusate, a modified Krebs-Henseleit bicarbonate buffer containing 25 mM glucose is pumped through an aortic cannula, entering the coronary arteries at the base of the aorta, and perfuses the tissues of the beating heart. The hearts were washed for 15 min at a flow rate of 30 ml/min in a non-recirculating mode, then the perfusion apparatus was altered to a recirculating mode and 4 mCi of (^{32}P)were added to a total perfusion volume of 40 ml of oxygenated buffer/heart. The perfusions were carried out at 37 C for 1-2 hrs depending on the vigor of the beating heart.

Upon termination of the perfusion period, the hearts were removed from the cannula, minced with scissors to 5 mm^2 pieces in Buffer B(50 mM Tris, pH 7.6, 250 mM KCl, 12.5 mM $MgCl_2$, 5 mM EDTA, 250 mM sucrose), and homogenized in a tissue homogenizer. This suspension was used for ribosome isolation.

Preparation of Purified Ribosomes

The cells obtained from either procedure were lysed by addition of Triton X-100 detergent (final concentration 0.5%) and homogenized with 6 strokes in the tissue homogenizer. The material was then centrifuged at 15,000 x g for 10 min. The supernatant was centrifuged at 105,000 x g for 2 hrs. The supernatant from this step was aspirated off, and the pellet suspended in Buffer C (20 mM Tris, pH 7.8, 80 mM KCl, 12.5 mM $MgCl_2$) containing 0.25 M sucrose. Brij-58 and sodium deoxycholate were added sequentially with gentle homogenization to a final concentration of 0.5% and 1.0% respectively. After centrifugation at 15,000 x g for 10 min to remove particulate matter, the supernatant was layered over Buffer C containing 0.5 M sucrose in a 1:1 (v/v) ratio. The supernatant generated from centrifugation at 144,000 x g for 2.5 hrs was carefully aspirated off from the top to avoid pellet contamination by detergents. The pellet is designated as the purified ribosomal pellet. All procedures in this section were carried out in ice or at 4 C.

Isolation of Proteins from Purified Ribosomes

The purified ribosomal pellet was resuspended in Buffer D (50 mM Tris, pH 7.8, 0.72 M KCl, 6 mM $MgCl_2$, 0.1 mM EDTA). This suspension was shaken in an ice bath for 30 min on a wrist shaker at a setting of 5. The resulting material was centrifuged at 144,000 x g for 2 hrs. The resulting supernatant is the Salt-Wash protein fraction and the pellet contains the Salt-Washed Ribosomes. Salt-washed ribosomal proteins were obtained by acetic acid extraction of these salt-washed ribosomes according to the method of Sherton and Wool (1972) with the following modifications: $MgCl_2$ was substituted for $Mg(OAc)_2 \cdot 4H_2O$ and succinate (3 mM) was added. All steps were performed in ice or at 4 C.

Analysis of (^{32}P)-labeled Phosphoproteins

Phosphoproteins were analyzed by electrophoresis on SDS-polyacrylamide gels by the method of Weber and Osborn (1969). Following electrophoresis on

disc gels, the gels were sliced into 2 mm slices and the slices were solubilized in 0.2 ml H_2O_2 for 16 hrs at 55 C. Radioactivity was measured by addition of Bray's scintillation cocktail and subsequent liquid scintillation spectrometry.

Specific activity of (^{32}P)-labeled proteins was determined by precipitation of protein solutions which were applied to Whatman 3 paper discs followed by soaking the discs in 5 ml ice-cold 10% TCA for 5 min. The washing procedure was repeated. The discs were then washed 2X each in 5 ml chloroform:methanol (2:1) at room temperature for 10 min to remove any phospholipids. Subsequent treatment of discs 2X for 5 min each in 5 ml of 5% TCA at 95 C hydrolyzes any labeled nucleic acid material.

Paper discs were then treated twice with each of the following solutions: 5 ml of 95% ethanol, ethanol:ether (3:1), and ether to remove any TCA. Radioactivity was determined by liquid scintillation spectrometry in Bray's solution.

Incorporation of (^{3}H)-leucine was determined by similar procedure but omitting the chloroform:methanol and hot 5% TCA steps.

Protein determinations on cell suspensions were by the method of Lowry, *et al.*, (1951) after solubilization in 0.5 M NaOH. Protein determinations on isolated SW or SWR proteins were by the method of Bradford (1976).

Histological Studies

Cross-sectional tissue slices were taken from both left and right venticle, imbedded in paraffin fixed on microscope slides, stained with H & E stain and examined under a phase contrast microscope. Cell counts were made within the borders of a photomicrograph attached to the microscope. Each slice was counted a minimum of 3 times at 430X magnification at randomly selected sites of the slice. Coordinates of each count were noted and recorded.

RESULTS AND DISCUSSION

Prior to studying the changes in ribosomal protein phosphorylation in developing cardiac hypertrophy, establishment of a hypertrophic state was necessary. Several parameters were examined which have been consistently associated with developing cardiac hypertrophy. Incorporation of (^{3}H)-leucine into acid insoluble material in cell suspensions of tissue obtained from control and treated animals was measured to provide a biochemical indication of changes in the rate of protein synthesis. As shown in Table 1, the level of (^{3}H)-leucine incorporation was elevated by 85-100% in experimental animals. However, these data do not indicate whether the higher levels of (^{3}H)-leucine incorporated into acid insoluble material in the treated animals is due to an increase in synthesis or a decrease in turnover. Addition of thyroxin or isoproterenol to incubations containing cells from untreated animals did not enhance the level of (^{3}H)-leucine incorporation. Other workers indicated that the major alterations in the basal protein synthetic activity in the hypertrophic heart are in the amounts of myofibrillar and mitochondrial proteins synthesized (Rabinowitz and Zak, 1972). Presumably the case is the same for this system although the actual fate of the labeled amino acid has not been determined here.

TABLE 1
Criteria of Hypertrophy

Inducing Agent	Cell Suspension Incubation[1] Dosage (mg/kg Body Weight)	^{3}H-Leu Incorp. E/C[2]	Ht. Wt./B.Wt. E/C
Thyroxin[3]	0.43	1.89	1.09
	1.00	1.99	1.15
	2.00	1.85	1.19
Isoproterenol[4]	2.50	2.04	1.31

[1]Experimental animals were injected with thyroxin or isoproterenol (thyroxin-2 mg/ml in 0.9% saline; isoproterenol - 5 mg/ml in 0.9% saline) at indicated doses for the indicated time periods. Control animals received 0.9% saline at the same ratio body wt/vol on the same schedule. Upon dispatch, heart weights (wet) were recorded after rinsing and pressing dry on tissue paper. Cell suspensions and levels of ^{3}H-leucine incorporation were obtained according to "Materials and Methods".

[2]E = Incorporation of (^{3}H)-Leu into acid insoluble material from experimental animals as analyzed by liquid scintillation spectrometry.
C = Incorporation of (^{3}H)-Leu into acid insoluble material from control animals as analyzed by liquid scintillation spectrometry.

[3]Subcutaneous injection at 24 hr intervals for 48 hrs.

[4]Subcutaneous injection at 12 hr intervals for 72 hrs.

A physiologic indication of hypertrophy was provided by measurements of the ratio of heart weight (wet) to total body weight of the animal (Table 1). Increases in this ratio in the thyroxin-induced hypertrophy appeared to be dose dependent, with a maximum increase of 19% at a dose of 2 mg/kg. Isoproterenol elicited a 30% increase at 2.5 mg/kg, thus both treatments appeared to satisfy this criterion.

Histological sections from hearts of control and treated animals were also examined for actual increases in cell volume. As shown in Table 2, a 32% decrease in cell counts of thyroxin (2 mg/kg) treated tissue relative to control was observed. Thus we can extrapolate that there was an increased cellular volume in the treated tissues. Isoproterenol treatment induced a 23% decrease in cell counts and also supported the existence of cellular hypertrophy in these cardiac tissues. No detectable gross morphological alterations in the cells were observed.

Analysis of ribosome-derived protein fractions indicated alterations in a) levels of protein phosphorylation and b) species of phosphoproteins in both the SW protein and SWR protein in treated compared to control tissue. The specific activities of the SW protein fractions indicated a higher (66%) level of protein labeling by (^{32}P) in the control tissue than in the heart tissue from treated animals (Table 3). An opposite trend was observed for the SWR proteins, *viz.* the specific activity of the control proteins was 6-fold lower than that of the treated.

TABLE 2

Histological Determinants of Hypertrophy

Animal	Inducing Agent	Dosage (mg/kg Body Wt.)	Term 1 inj/24 hrs (48 hrs)	Average Cell Count L & R (cells/field)
Exp. IV				
A	--	--	"	43.8 ± 4.5
B	--	--	"	46.0 ± 0.5
C	Thyroxin	1	"	41.0 ± 0.9
D	"	1	"	36.0
E	"	2	"	27.0 ± 1.0
F	"	2	"	34.0 ± 0.5
Exp. VII			1 inj/12 hrs (120 hrs)	
A	Isoproterenol	3		33.0 ± 1.1
B		3		36.0 ± 6.2

	% Control		
Thyroxin	Avg. Cell Count	% Decrease	
Control	45.0	14.4	32
1 mg/kg body wt.	38.5		
2 mg/kg body wt.	30.5	20.7	
Isoproterenol			
Control	45.0	23%	
3 mg/kg body wt.	35.0		

[1]Tissue slices were prepared according to "Materials and Methods." Slices were examined under phase contrast microscope. Cells falling within borders of a photomicrograph field attached to microscope were counted. Coordinates of the areas were noted and recorded for future reference. Slide counting was done randomly on slides taped to conceal sample and treatment conditions. These values represent the average of 3 or more independent determinations.

TABLE 3

Specific Activity of Phosphoprotein

Origin of Phosphoprotein	Specific Activity[1] (CPM/Mg) Control	Experimental
Ribosome-Associated	15,000	9,000
Ribosomal	800	5,000

[1]A known amount of protein (determined by the Coomassie blue method) was spotted on a paper disc and ^{32}P specific activity was determined as described in "Materials and Methods".

That the radioactivity indeed resided in the proteins and not in either phospholipids or nucleic acids is shown in Table 4. The ribosome-associated protein appears to lose negligible amounts of (^{32}P) upon treatment by this method and probably contains no phospholipid or RNA.

The molecular weight of the phosphoproteins was determined by electrophoresis on SDS-polyacrylamide gels. Results from scintillation counting of solubilized gel slices are presented in Table 5. Radioactivity appeared in protein bands of approximately 60 K, 45K, 36K and 10K daltons in the control SW protein fraction. The salt-wash protein from the hypertrophic tissue, however, contained (^{32}P) in 2 bands essentially, 36K and 25K, the former band being most heavily labeled. The disappearance of labeling from several species of SW protein could account for the decrease in specific activity of the treated fraction as compared to the control fraction.

TABLE 4

Solubility Characteristics of ^{32}P-Labeled Salt-Washed Ribosomal Proteins and Salt Wash Proteins[1]

Procedure	% Control
Salt-Washed Ribosomal Protein	
A) Cold 10% Trichloroacetic Acid	100
B) $CHCl_3$:MeOH (2:1)	5 ± 2.0
C) Hot 5% Trichloroacetic Acid	13 ± 1.6
D) Residue	80
Salt-Wash Protein Fraction	
A) Cold 10% Trichloroacetic Acid	100
B) $CHCl_3$:MeOH (2:1)	100
C) Hot 5% Trichloroacetic Acid	100
D) Residue	100

[1]Protein of known specific activity was spotted and dried on paper discs. Discs were subjected to single treatments of A, B, or C or all treatments sequentially. Discs were ethanol-ether treated to remove TCA and counted by LSC in Bray's cocktail.

The SWR proteins from the control were labeled in 2 protein bands of 57K and 20K daltons. This contrasts the labeling in the hypertrophic heart which usually resides in bands of approximating molecular weights of 42K, 36-38K, and 20K daltons.

In order for phosphorylation of endogenous proteins with exogenously added (^{32}P)-orthophosphate to occur, the polypeptide must contain site(s) which are susceptible to a corresponding protein kinase. One might expect these sites to exist in their phospho- or dephospho- forms (as dictated by the tissue metabolic

state) previous to the addition of exogenous label in the incubation medium or perfusate. To attain the levels of phosphorylation observed in these data during 2 hrs of perfusion or incubation, the phosphate groups, or even proteins (in the case of the SW protein) must be turning over at a significant rate. The SWR and SW protein may also contain phosphoproteins which have a lower turnover rate hence might not be labeled during this short period. However, apparently there exists some process by which (^{32}P) is associated with ribosomal and ribosome associated proteins in substantial amounts.

TABLE 5[1]

Origin	Approximate Molec. Wt. ($x10^3$)	Phosphorylation Experimental	Control
Salt-Wash Protein	60 ± 3	–[2]	+[3]
	45 ± 4	–	+
	36 ± 4	+	+
	20 ± 4	+	–
	10	–	+
Salt-Washed Ribosomal Protein	57	–	+
	42	+	–
	36 ± 2	+	–
	20 ± 4	+	+

[1]Proteins were isolated according to "Materials and Methods". Molecular wts were determined by SDS-polyacrylamide disc gel electrophoresis according to Weber and Osborn (1969). Gels were sliced into 2 mm lengths and the slices solubilized for 16 hrs at 55 C in 0.2 ml H_2O_2. Solubilized gel slices were then counted by LSC after addition of Bray's cocktail.

[2]absence of ^{32}P-radioactivity.

[3]presence of ^{32}P-radioactivity.

Identification of our labeled proteins at this point is purely speculative. It is interesting to note the correspondence in size between the previously mentioned S-6 phosphoprotein of the small ribosomal subunit from rat liver (36K daltons) and from other systems. Phosphoproteins have also been identified from the salt-wash protein fraction from rabbit reticulocytes which appear to be protein subunits of eukaryotic initiation factors. Covalent modification of these factors by corresponding kinases has been shown to affect there catalytic activities relating to the translational level of protein synthesis. One such polypeptide has a molecular weight of approximately 36K daltons and has been implicated in the initiation complex formation activity of eIF2. There are other reports that eIF3 may also act as substrate for kinase enzymes *in vivo* in rabbit reticulocytes (Benne, *et al.*, 1978). Attempts at identification of phosphorylated SW protein and SWR protein by gel electrophoresis are in progress.

CONCLUSIONS

Phosphorylation of salt-washed ribosomal proteins and salt-wash protein fractions in the presence of (^{32}P)-orthophosphate has been demonstrated in the isolated rabbit heart as it has in other eukaryotic systems. Alterations in this phosphorylation can be induced by pretreatment of the animal with thyroxin or isoproterenol. This treatment induces cardiac hypertrophy in the rabbit and this condition has been substantiated by a number of methods: a) biochemically, b) physiologically and c) histologically. The changes in (^{32}P)-labeling appear to be intimately associated with the metabolic activity of the protein synthetic machinery in the myocardial tissue. One major goal is to correlate all these changing parameters of the development of hypertrophy in the rabbit cardiac tissue.

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PLANT MEDIATED ACTIVATION OF MUTAGENS

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ABSTRACT

Various microbial test systems which respond in a positive manner to liquid 1, 2-dibromoethane (EDB) exposure exhibited a negative mutagenic response when this mutagen was delivered in a gaseous form. These observations have lead to the hypothesis that this compound induces genetic alterations by 2 routes. One route apparently involves "breakdown" products *in vitro* which are active in liquid exposures and a second route which involves metabolic activation by plants.

These results indicate the need for further studies on plant metabolism of environmental chemicals (pesticides) for a clearer understanding of the potential genetic hazards to man, either directly by ingestion or indirectly by integration into the ecosystem.

INTRODUCTION

1, 2-dibromoethane (EDB) is widely used as a pesticide, fumigant, and as an additive to petrol (FAO commission, 1965; Girish, *et al.*, 1972). In the gaseous phase, at a level of 1 part/million (ppm), this compound induced mutation in stamen hair cells of *Tradescantia* species (Nauman, *et al.*, 1976; Sparrow, *et al.*, 1974; Underbrink, *et al.*, 1973). However, at a dose level 100 times this concentration various microorganisms exposed on Millipore filters and subsequently assayed for mutation exhibit a negative response (Table 1). This observation is at variance with the available results of dose equivalent liquid EDB exposures, using the same assay systems, which were positive (Table 1). Such variances in response could be due to a differing sensitivity of the various assay systems; alternatively, it could indicate that this mutagen induces mutation by 2 reaction pathways. The first involving a "breakdown" *in vitro*, and the second involving metabolic activation by plants.

[1] It is with deepest regrets that we acknowledge the untimely death of our colleague and friend, on June 25, 1976. His breadth of scientific interest and expertise and untiring efforts in support of plant science will be greatly missed by his colleagues throughout the world.

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TABLE 1

A Comparison of the Deleterious Effects (Inactivation and Mutagenic) of EDB Liquid and Gaseous Exposures (+ = Positive and - = Negative Response)

Biodetector	Liquid Exposure	Gaseous Exposure
Escherichia coli	+(Ames and Yanofsky, 1971; Kristofferson, 1974)	-
Salmonella typhimurium	+(Ames and Yanofsky, 1971)	-
Neurospora crassa	+(De Serres, and Malling, 1970; Malling, 1969)	-
Aspergillus nidulans	+(Scott, *et al.*, 1978)	-

The purpose of this investigation was to confirm the mutagenicity of EDB in liquid tests and determine whether the addition of plant mixtures to the microbial cultures would elevate the levels of mutagenicity detected. For brevity, only representative results obtained with the methionine system of *Aspergillus* and the tryptophan system of *E. coli* are reported here (A fuller form is reported in Scott, *et al.*, 1978).

MATERIALS AND METHODS

The Aspergillus System

The methionine system in *Aspergillus* detects prototrophic revertants arising from forward mutation at any of 6 different suppressor loci (Lilly, 1965; Scott and Alderson, 1971).

Preparation of conidial samples: Each experiment used conidia from a single colony of the *methG1 biA1* (requiring methionine and biotin) Glasgow strain of *Aspergillus nidulans*, grown for 5-6 days on complete medium (Scott, *et al.*, 1972), at 37 C. Inhibitor-depleted conidia (Scott, *et al.*, 1972) were prepared by the addition of a 0.1% (V/V) solution of diethyl ether to the phosphate buffer (pH 7.0) as previously reported (Scott and Alderson, 1974).

Mutation and viability platings: Techniques used to assess the conidia viability (Scott, *et al.*, 1972) and reveal mutagenic effects (Scott, *et al.*, 1973) were as previously described. However, Difco Purified Agar replaced Oxoid No. 3 in the present experiments. Immediately after treatment samples of conidia were plated onto solid medium, the surface of which had been dried until crinkled, to prevent any uncontrolled delayed plating (liquid holding) effect. The methionine revertants observed after 5-6 days incubation of the methionine-less plates were classified as A (large green colonies), B (heavily pigmented brown colonies) or C (small green colonies with a white hyaline edge) in accordance with the system described by Lilly (1965).

E. Coli WP2 System

E. coli WP2 try⁻ mutates to prototrophy by either the induction of true reverse mutations at the tryptophan locus or ochre suppressors elsewhere in the genome (Bridges, *et al.*, 1967a, 1967b; Law, and Scott, 1975).

Culture conditions and assay method: Bacteria were grown overnight to a density of about 2 x 10^9 cells/ml in nutrient broth supplemented with 5 g/l of NaCl, harvested by centrifugation at 6000x g (15 min at -4 C) and resuspended in an equal volume of ice-cold phosphate buffer (pH 7.0). After repeating this procedure 3 times, the "stationary" cells were finally resuspended at a density of approximately 1 x 10^{10} cells/ml in phosphate buffer. Following the appropriate treatment of these cells, mutant and viable bacteria were estimated after appropriate dilution on glucose-salts minimal medium agar supplemented with 0.075 μg/ml and 0.75 μg/ml tryptophan, respectively (Bridges, *et al.*, 1967a, 1967b).

Treatment with EDB, EMS and Preparation of Crude Plant Extract

For each experiment a similar procedure was followed. Initially, various combinations of EDB (0.1 ml of a mixture of 0.8 ml EDB, and 4.2 ml DMSO), EMS (0.1 ml), DMSO (0.1 ml of a mixture of 4.2 ml DMSO and 0.8 ml of H_2O), a phosphate buffer pH 7.0 (0.1 ml to 3.1 ml in a different combination), plant extract (2 ml of the supernatant obtained using the protocol outlined in Figure 1),

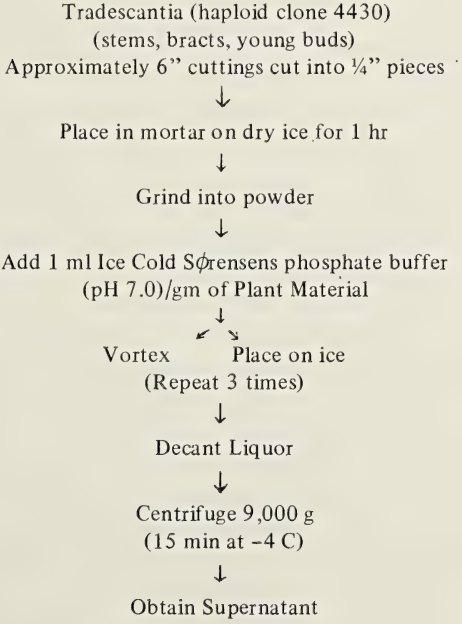
Tradescantia (haploid clone 4430)
(stems, bracts, young buds)
Approximately 6" cuttings cut into ¼" pieces
Place in mortar on dry ice for 1 hr
Grind into powder
Add 1 ml Ice Cold Sørensens phosphate buffer
(pH 7.0)/gm of Plant Material
Vortex
Place on ice
(Repeat 3 times)
Decant Liquor
Centrifuge 9,000 g
(15 min at -4 C)
Obtain Supernatant

Figure 1. Preparation of S9 extract.

NADPH (1 ml of a freshly prepared solution, concentration 3.5 mg/ml) and the appropriate microbe (1 ml) were made up in 125 ml stoppered Erhlenmeyer flasks. These were then placed in an environmental chamber at 37 C and agitated for 30 min or 3 hrs for the *E. coli* and *A. nidulans* experiments, respectively (flasks containing the EMS treatments were agitated for only 30 min in both cases). After exposure, the contents of the various treatment and controlled flasks, together with a 10 ml ice-cold phosphate buffer wash (pH 7.0), were filtered individually through a 0.22 μm filter. The material collected was washed with 20 ml of ice-cold phosphate buffer and finally resuspended in 4.1 ml of the same buffer. This suspension was then treated as described elsewhere.

RESULTS AND DISCUSSION

Although the genetic activity of the compound known as EDB in the liquid phase was confirmed for *E. coli* and *Aspergillus*, it was also indicated that high boiling point impurity (greater than 131 C, the boiling point of EDB) might be the cause of such an effect. This is suggested by the observation that the residue remaining behind after distillation exerts a greater inactivation or mutation effect than the pure distillate (Fig. 2). The nature of such a possible impurity is as yet unknown but it can be observed from this figure that as the age of the distillate increases, so does the genetic effectiveness of the "compound." Such a product, when the phase of the EDB changes from a liquid to a gas, would remain behind in the solution because of the indicated high boiling point of the impurity, and if this were the cause of the observed genetic effect in liquid exposures no effect would be expected to be observed in the gaseous exposures. However, this does not explain why *Tradescantia* stamen-hair cells are mutated after exposure of the influorescences to pure gaseous EDB (Nauman, *et al.*, 1976; Sparrow, *et al.*, 1974), unless the plant is capable of converting the EDB to an active mutagen. If this were the case, it might be expected that assay systems, capable of detecting this mutagen under liquid exposure conditions in the presence of plant extract would exhibit an elevated response. The results presented in Table 2, Figs. 3 and 4 demonstrate such a response. Also, because the degree of mutagenicity induced by the EMS is depressed in the presence of the plant mix (plant extract plus NADPH) (Figs. 3 and 4), this effect apparently is not due to synergistic reaction of a mutagen and the plant mix.

At the present moment, it is unknown whether such a reaction involves the soluble enzyme fraction or the microsomal fraction of the S9 supernatant (microsomes remaining in suspension at 9000x g). However, an experimental accident in which NADPH was omitted from the plant mix reaction gave no increase in the yield of mutants detected. This indicates that, in part, this response must be enzymatic in nature because NADPH is required for some enzymatic reactions. Furthermore, evidence that EDB can be metabolically converted to other substances has been reported by Edwards, *et al.*, (1969, 1970). In these studies of

injecting EDB into mice and rats, it was found that EDB is not excreted unchanged but metabolized mainly to S-(2-hydroxyethyl)-cysteine and its N-acetate derivatives; no *in vivo* reactions of "sulphur-stripping" were detected. This may also indicate that at least 2 reaction pathways for this compound are possible, one similar to the *in vitro* reaction ("sulphur-stripping") and the second involving metabolic activation.

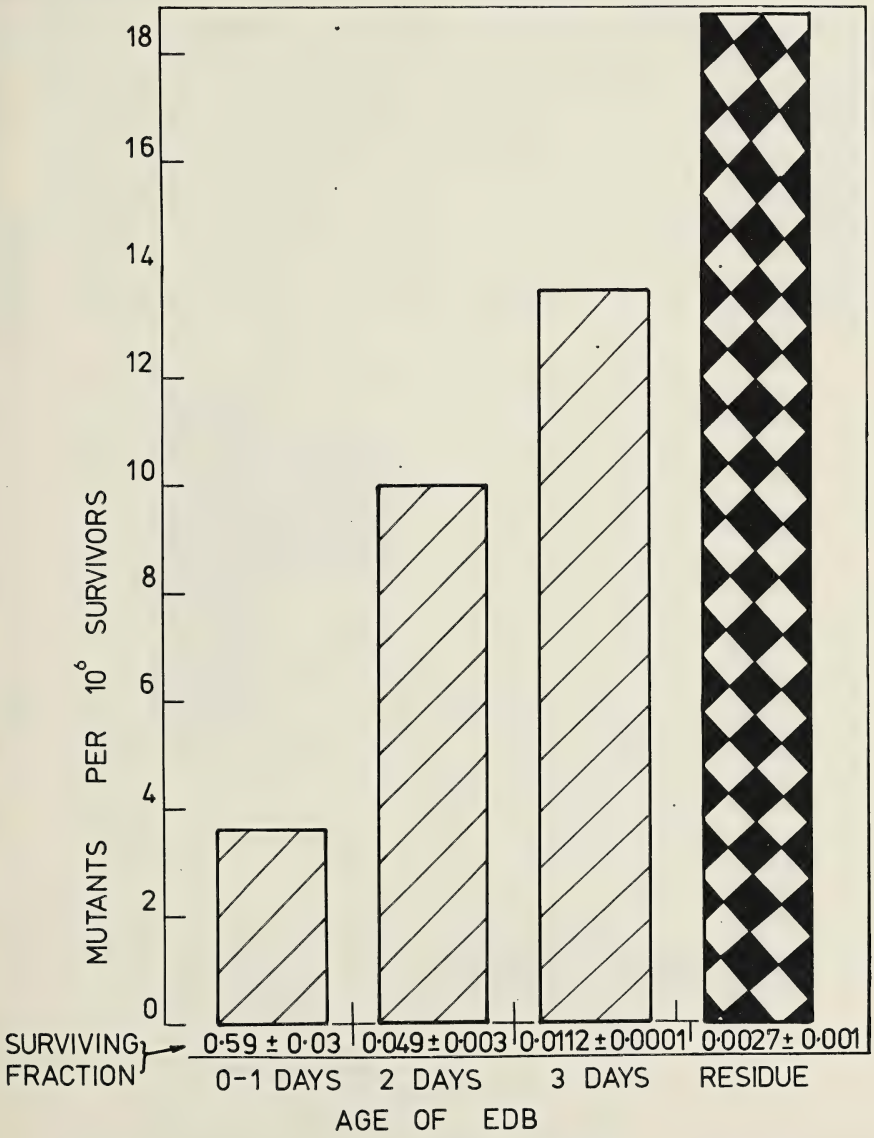

Figure 2. The inactivation and genetic reversion of *E. coli* WP2 try$^-$ after treatment with EDB of various ages and the residue remaining behind after distillation of EDB.

TABLE 2

Representative Experimental Results of the Inactivation of *E. coli* WP2 try⁻ and *A. nidulans* after Treatment with EDB, EMS in the Presence or Absence of Plant Mix (Extract + NADPH) and the Respective Controls

Treatment	Surviving Fraction ± Standard Error	
	E. coli	*Aspergillus nidulans*
1. EDB	0.0112 ± 0.0001	0.53 ± 0.03
2. EDB + Plant Mix	0.0021 ± 0.0002	0.27 ± 0.02
3. EMS	0.5600 ± 0.0400	0.49 ± 0.07
4. EMS + Plant Mix	1.1900 ± 0.1200	0.68 ± 0.05
5. DMSO Control	1.0200 ± 0.0700	1.13 ± 0.16
6. DMSO + Plant Mix Control	0.9700 ± 0.0600	1.05 ± 0.11
7. Plant Mix Control	0.9900 ± 0.0600	0.91 ± 0.06
8. Buffer Control	1	1

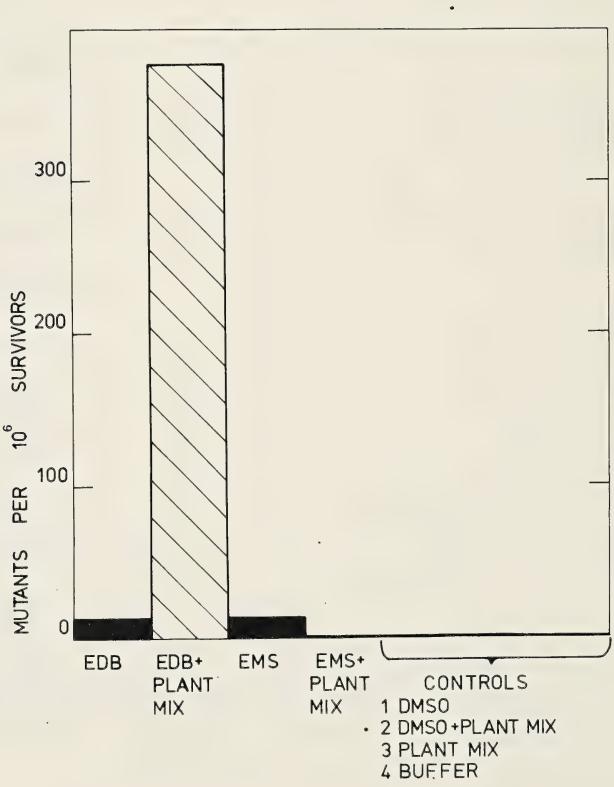

Figure 3. The genetic reversion of *E. coli* WP2 try⁻ after treatment with EDB, EMS in the presence and absence of plant mix (extract + NADPH) and the respective controls.

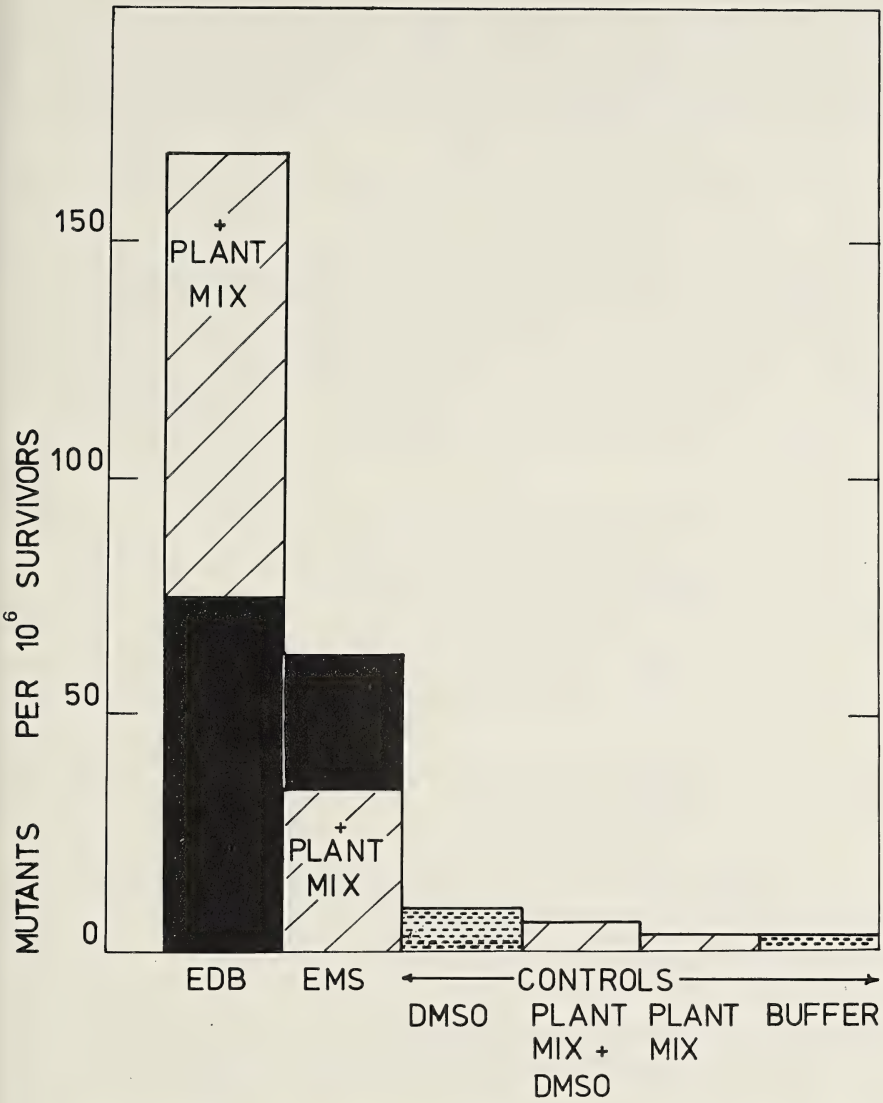

Figure 4. The effect of treatment of *Aspergillus* conidia on genetic reversion (by suppressor mutation at various loci of a *meth G 1* strain) with EDB, EMS in the presence or absence of plant mix (extract + NADPH) together with the respective controls.

CONCLUSION

It appears that EDB is capable of being converted to a mutagen by 2 reaction pathways, one involving a breakdown product *in vitro*, and the other involving

metabolic activation by plants. These experiments raise a series of questions, the most urgent of which, from a human health point of view, is, do these metabolically formed mutagens persist in plant material? Indication that at least one agricultural chemical persists in maize has been demonstrated by Plewa and Gentile (1976) using the mutagen atrazine.

ACKNOWLEDGEMENTS

We would like to thank Dr. F.J. de Serres for his encouragement and help and Juanita Davis, Nancy Brooks, Jenny Foushee, Catherine White and Mike Spillane for preparation of the media. The assistance of Lynda Scotti and Richard C. Sautkalis with some of the experiments is acknowledged. Drs. Tom Clemmer, Bahadur Singh and Janet Anderson are also thanked for their calculation of the results using a computer program developed by them.

COMMENTS

Sharma: You mentioned that rats and mice metabolize EDB, but is there any evidence that these products are mutagenic or carcinogenic.

Scott: In addition to the report by Olson, *et al.*, (1973) that EDB induces tumors in mice, preliminary evidence with rat microsomes and EDB would indicate that animals can metabolize EDB to an agent of greater potency similar to that found in plants.

Nairn: Are there any other promutagens metabolized by plants besides the ones which you have mentioned?

Scott: The only other mutagen which we have tested under these conditions is Aflatoxin B 1. Again, with the *Aspergillus* methionine system and plant mix, this compound gives a positive mutagenic response, whereas in the absence of the plant extract and NADPH the response is negative.

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COMPARISON OF THE METABOLISM OF ARABINOSYLADENINE AND ARABINOSYL-2-FLUOROADENINE BY HUMAN LEUKOCYTES

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ABSTRACT

The cytotoxicity and metabolism of arabinosyladenine (ara-A) in the absence and presence of deoxycoformycin (dCF), an adenosine deaminase inhibitor, by human lymphoblastoid cells (line CCRF-CEM) and PHA-stimulated human lymphocytes has been determined and compared to that of arabinosyl-2-fluoroadenine (F-ara-A). The inclusion of dCF increases the toxicity of ara-A in each system. F-ara-A, which is not a substrate for adenosine deaminase, has the same toxicity and inhibitory activity toward DNA synthesis in CCRF-CEM cells as ara-A plus dCF, but is substantially less effective in inhibiting DNA synthesis in PHA-stimulated lymphocytes. Since the active nucleoside triphosphates, araATP, are accumulated to similar concentrations by cells of each system, these results suggest a differential sensitivity of the DNA synthetic machinery of CCRF-CEM cells and PHA-stimulated lymphocytes.

INTRODUCTION

Arabinosyladenine (ara-A) is an effective antiviral agent in humans (Whitely, *et al.*, 1977). Clinical trials have demonstrated that ara-A also possesses promising anticancer activities (LePage, *et al.*, 1972; LePage, *et al.*, 1973; Bodey, *et al.*, 1975). However, the chemotherapeutic effectiveness of ara-A is limited by its deamination to the far less active arabinosylhypoxanthine (ara-Hx) by adenosine deaminase (Hubert-Habart and Cohen, 1962; Koshiura and LePage, 1968; Plunkett and Cohen, 1977). Direct inhibition of adenosine deaminase by either *erythro*-9-(2-hydroxy-3-nonyl) adenine (EHNA) (Schaeffer and Schwender, 1974) or by 2'-deoxycoformycin (dCF) (Woo, *et al.*, 1974) greatly increases the cytotoxicity (Plunkett and Cohen, 1975; Cass and Au-Yeung, 1976) and the antitumor activity (Plunkett and Cohen, 1975; LePage, *et al.*, 1976; Schabel, *et al.*, 1976; Lee, *et al.*, 1977) of ara-A. These increases in the therapeutic activity of ara-A in the presence of deaminase inhibitors are correlated with elevated cellular levels of the active triphosphate form, ara-ATP, and a decrease in

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the DNA synthetic capacity of the target cells (Plunkett and Cohen, 1977; Rose and Brockman, 1977; Plunkett, *et al.*, 1977; Plunkett, *et al.*, 1978).

A simpler approach to bypassing the deaminative inactivation of ara-A is to design an adenine nucleoside analogue that is not a substrate for adenosine deaminase, but retains the potent antitumor activity elicited by ara-A in the presence of a deaminase inhibitor (Montgomery and Hewson, 1969). Brockman, *et al.*, (1977) have demonstrated that F-ara-A is not a substrate for adenosine deaminase and is selective in inhibiting DNA synthesis but not RNA or protein synthesis as is ara-A (Doering, *et al.*, 1966). Furthermore, the antitumor activity of F-ara-A appears to be comparable to that of ara-A plus deoxycoformycin (Brockman, *et al.*, 1977). The present report compares the cytotoxic effect and the metabolism of F-ara-A with that of ara-A alone and in the presence of dCF by both a line of lymphoblastoid cells derived from a patient with acute lymphocytic leukemia and PHA-stimulated human lymphocytes in culture. The results suggest that F-ara-A may be a useful agent in human disease.

MATERIALS AND METHODS

A line of lymphoblastoid cells from a patient with acute lymphocytic leukemia (CCRF-CEM) (Foley, *et al.*, 1965) were obtained from the laboratory of Dr. E.M. Hersh of this Department. The cells were grown in medium RPMI-1640 supplemented with 10% fetal calf serum in suspension culture with constant agitation at 37 C. Under these conditions, the population doubling time was 20-22 hrs as determined by an electronic particle counter (Coulter Counter, Model B).

Human lymphocytes were obtained as follows. Blood from a healthy donor was drawn into a heparinized syringe, diluted with 4 volumes of phosphate-buffered saline, layered over a mixture of 7.8 ml 8% Ficoll and 2.5 ml 50% hypaque and centrifuged at 400 x g for 35 min at 21 C. Lymphocytes were removed from the interface and washed twice in incubation medium (Morgan, *et al.*, 1974) before they were counted and resuspended in pre-warmed drug-free medium at a final concentration 1 x 10^6 cells/ml. Morphologically, the final suspension was 97% small lymphocytes of which > 94% excluded trypan blue dye. DNA synthesis was stimulated and quantitated in these cells as follows. One half ml of the final cell suspension was added to a series of tubes containing phytohemagglutinin (PHA-P, Difco) and incubated in a 5% CO_2 - 95% air high humidity atmosphere at 37 C. On the indicated day, 2.5 μCi of [methyl-^{3}H]-thymidine (2-6 Ci/mmole) was added, incubated for 2 hrs and the incorporation of radioactivity into perchloric acid-insoluble material was determined. All points are the average of triplicate determinations ± SEM. When cells were treated with arabinosyl nucleosides, the drugs were added 4 hrs before thymidine labeling.

The metabolism of arabinosyl nucleotides was analyzed by high-pressure liquid chromatography. A Waters Associates ALC-204 high-pressure liquid chromatograph equipped with 2 Model 6000A pumps, a Model 660 Gradient programmer and a column (25 cm x 4.6 mm) containing Partisil-10 SAX anion exchange resin (Whatman, Inc.) was employed to fractionate perchloric acid-soluble extracts. Samples (1-5 x 10^6 cell equivalents) were injected by means of the U6K-LC injection system and eluted with either of 2 methods. The first used the linear gradient system of Nelson, *et al.*, (1976) as adapted by Rose and Brockman (1977) and Brockman, *et al.*, (1977) for the separation of arabinosyl nucleotides from normal cellular nucleotides (System I). The second employed a convex gradient from 0.150 M $NH_4H_2PO_4$, pH 3.7 to 0.675 M $NH_4H_2PO_4$, pH 3.7 over 50 min on gradient program number 5 or according to the equation $y = \sqrt{1000x}$ (System 2). The main advantage of the latter system was that the separation and resolution of normal cellular and arabinosyl nucleoside triphosphates achieved by System 1 was maintained, but required only 60% of the time. Eluted compounds were detected at 254 nm by the Model 440 detector and quantitated with a CDS-111 electronic integrator (Varian Associates).

RESULTS AND DISCUSSION

To determine the relative efficacy of F-ara-A with ara-A alone and in the presence of an inhibitor of adenosine deaminase, the growth of CCRF-CEM cells in the continuous presence of various concentrations of each compound was determined (Fig. 1). At 1.0 μM, F-ara-A has similar growth inhibitory effects as ara-A in the presence of 1 μM dCF. At this concentration, dCF alone is not inhibitory to cell growth. At 2.5 μM, F-ara-A and ara-A in combination with dCF is cytostatic. In contrast, ara-A alone at 25 μM is growth inhibitory but not as cytostatic. Thus, ara-A inhibition of cell growth is clearly limited by deamination, both by the cells and the deaminase activity in the fetal calf serum. This limitation is bypassed equally well by either the addition of an inhibitor of adenosine deaminase or by use of F-ara-A, a deaminase-resistant form of the analogue.

As shown in Figure 2 the accumulation of F-ara-ATP, the triphosphorylated metabolite of F-ara-A, may be clearly detected by high-pressure liquid chromatographic techniques. Incubation of CCRF-CEM cells with 100 μM F-ara-A results in the concentrative accumulation of F-ara-ATP (Fig. 3) When considering the average cell volume, the number of cell equivalents analyzed and the number of nmoles of F-ara-ATP detected, it is possible to calculate that after 4 hrs the cellular F-ara-ATP concentration was approximately 300 μM or 3 times the concentration of exogenous nucleoside. A much more marked concentrative effect was seen in separate experiments in which cells incubated with 10 μM F-ara-A for 28 hrs accumulated intracellular F-ara-ATP to 200 μM.

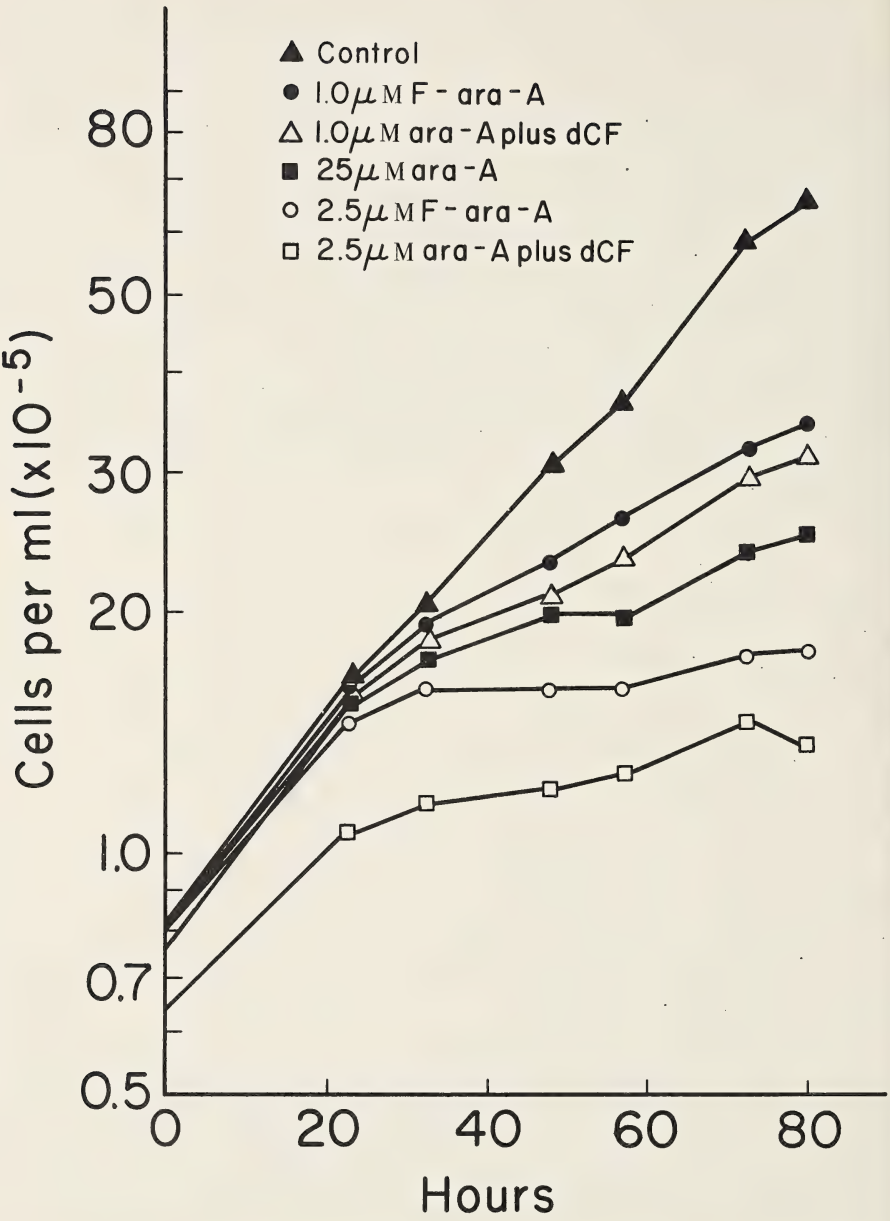

Figure 1. Effect of F-ara-A, and of ara-A, either alone or in the presence of 1 μM dCF on the growth of CCRF-CEM cells.

The effect of F-ara-A and ara-A either alone or in the presence of dCF on the incorporation of thymidine into acid-insoluble material by CCRF-CEM cells

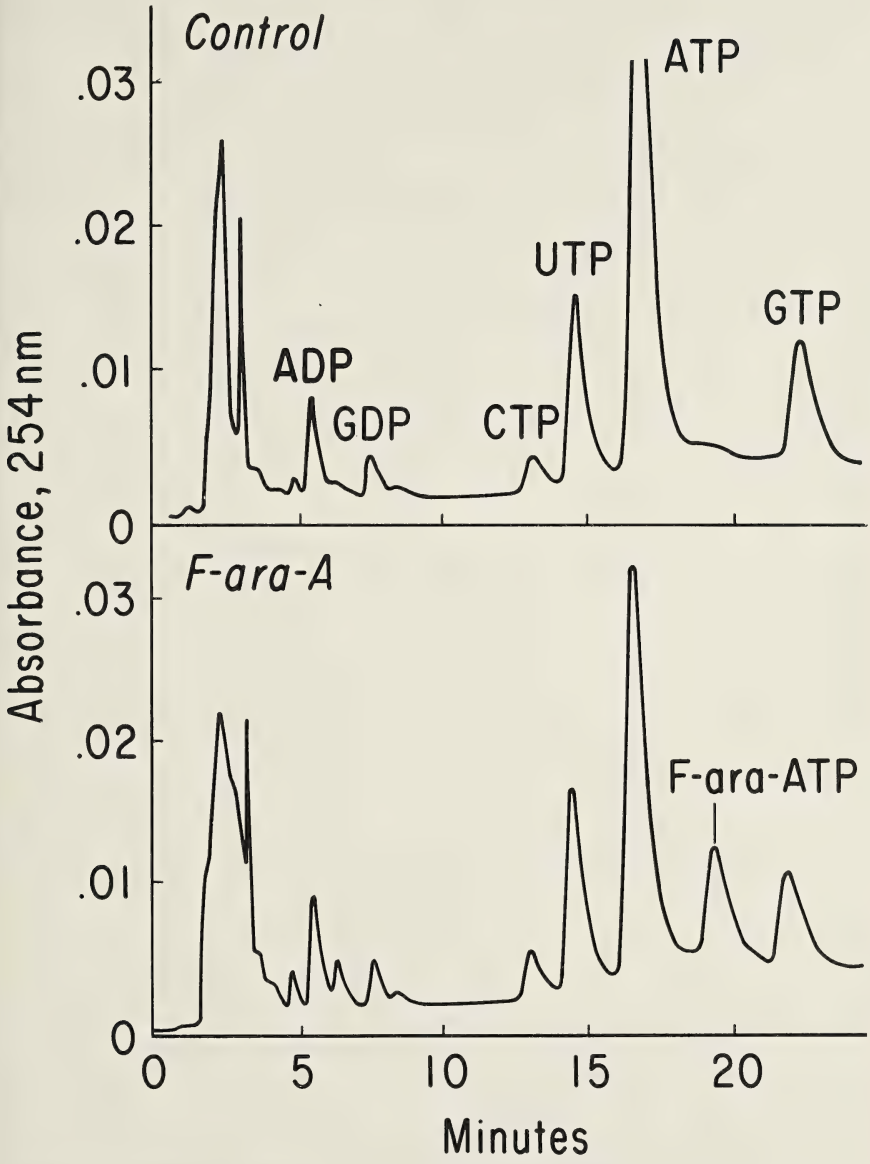

Figure 2. High-pressure liquid chromatographic analysis of perchloric acid-soluble material from 2.5 x 10^6 CCRF-CEM cells after a 4 hr incubation in the presence or absence of 100 μM F-ara-A. Solvent system 2 was used in this analysis.

is shown in Figure 4. After 1 hr of incubation with 100 μM F-ara-A, thymidine was incorporated at less than 1% of the control rate. Thymidine incorporation was inhibited to less than 10% of the control by ara-A either alone or in the

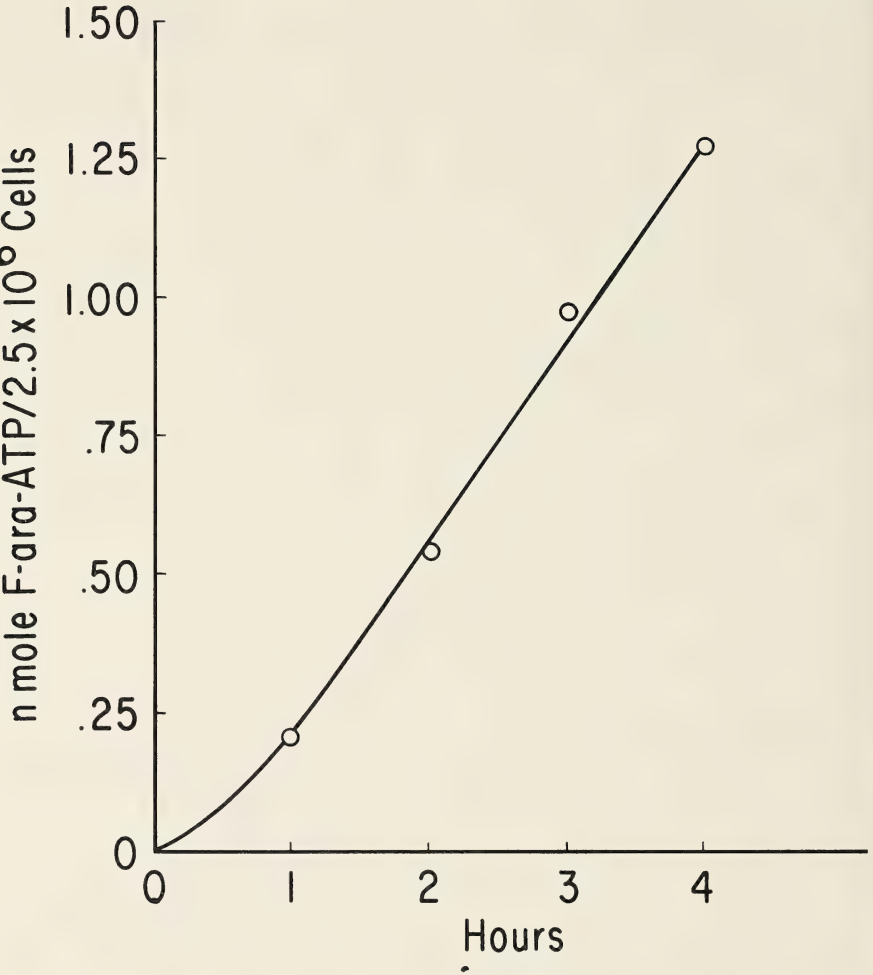

Figure 3. Accumulation of F-ara-ATP in CCRF-CEM cells during incubation with 100 μM F-ara-A. High-pressure liquid chromatographic analysis was carried out using solvent system 1.

presence of dCF, suggesting that the initial drug concentration exceeded that level which might be limited by deamination. In separate experiments, cells incubated for 4 hrs with either F-ara-A or ara-A plus dCF and then washed into drug-free medium, did not start to recover the ability to incorporate thymidine until 6 hrs after washing. Control experiments proved that at 1 hr neither F-ara-A nor ara-A in the presence of dCF significantly altered the specific activity of dTTP in the cells.

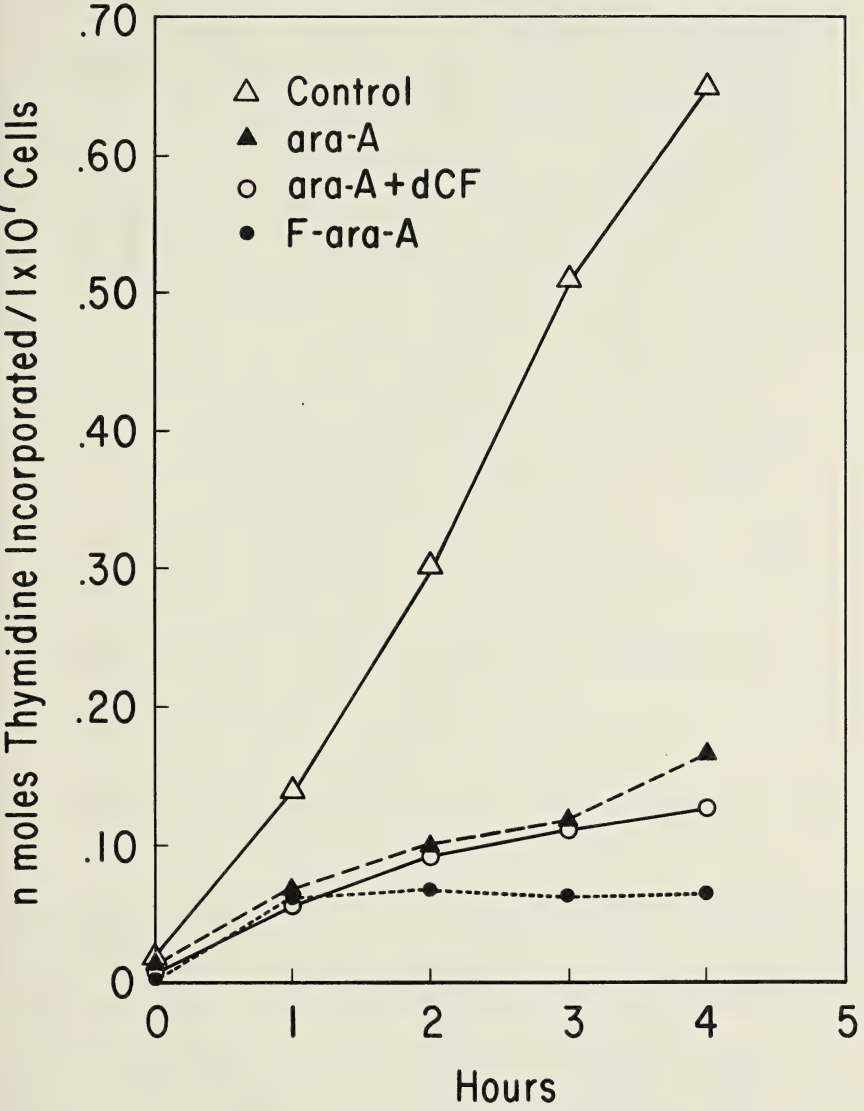

Figure 4. Effect of arabinosyl nucleosides on the incorporation of thymidine into perchloric acid-insoluble material by CCRF-CFM cells.

We have employed a different biological system to ask similar questions about the relative biological effectiveness of F-ara-A and ara-A. Human lymphocytes may be stimulated to synthesize DNA by incubation with PHA (Fig. 5). Maximum stimulation of thymidine into DNA occurred after 3 days of incubation.

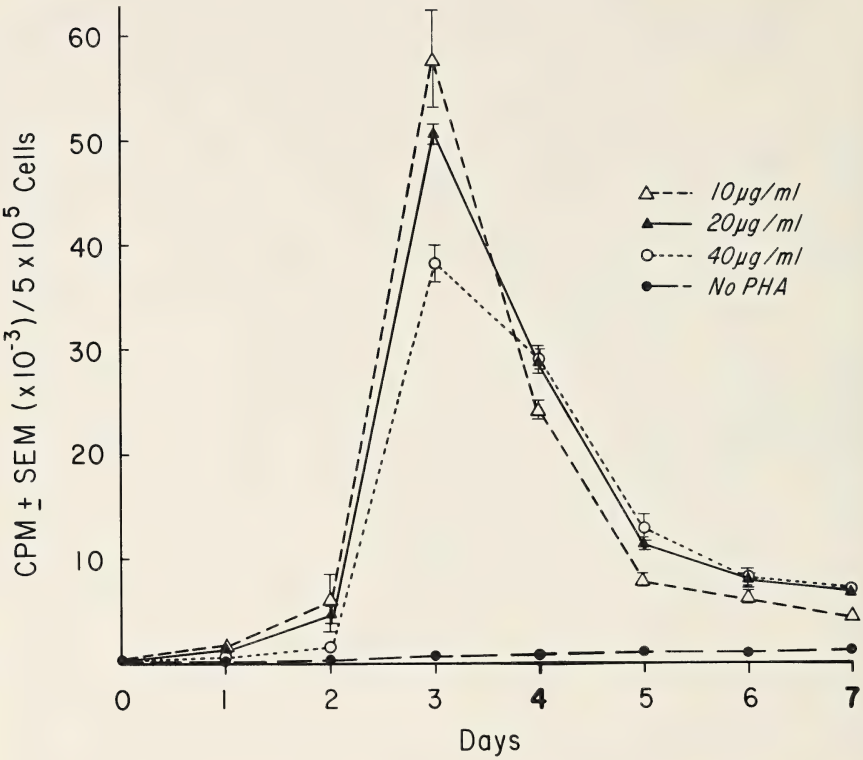

Figure 5. Incorporation of [^{3}H] thymidine by human lymphocytes incubated with different concentrations of PHA.

This stimulation was dependent upon the concentration of PHA used, 10 μg PHA/ml giving 88 times the incorporation of cells incubated in the absence of PHA. Subsequent experiments demonstrated that lesser amounts of PHA were not as effective as 10 μg PHA/ml, the concentration used in all subsequent experiments.

Figure 6 shows the effects of various concentrations of ara-A alone or in the presence of dCF (1 μM), and of several concentrations of F-ara-A on thymidine incorporation by PHA-stimulated lymphocytes. Ara-A was more inhibitory in the presence of dCF than when administered alone, the greatest differential in the inhibition with and without dCF coming at the lower ara-A concentrations. Since dCF alone at 1 μM had no inhibitory effect on thymidine incorporation, and the incubation medium contained horse serum which is devoid of adenosine deaminase (Ishii and Green, 1973) these results suggest an active role for the cellular adenosine deaminase in the inactivation of ara-A. Surprisingly, although insensitive to inactivation by deamination, F-ara-A was not inhibitory to thymidine

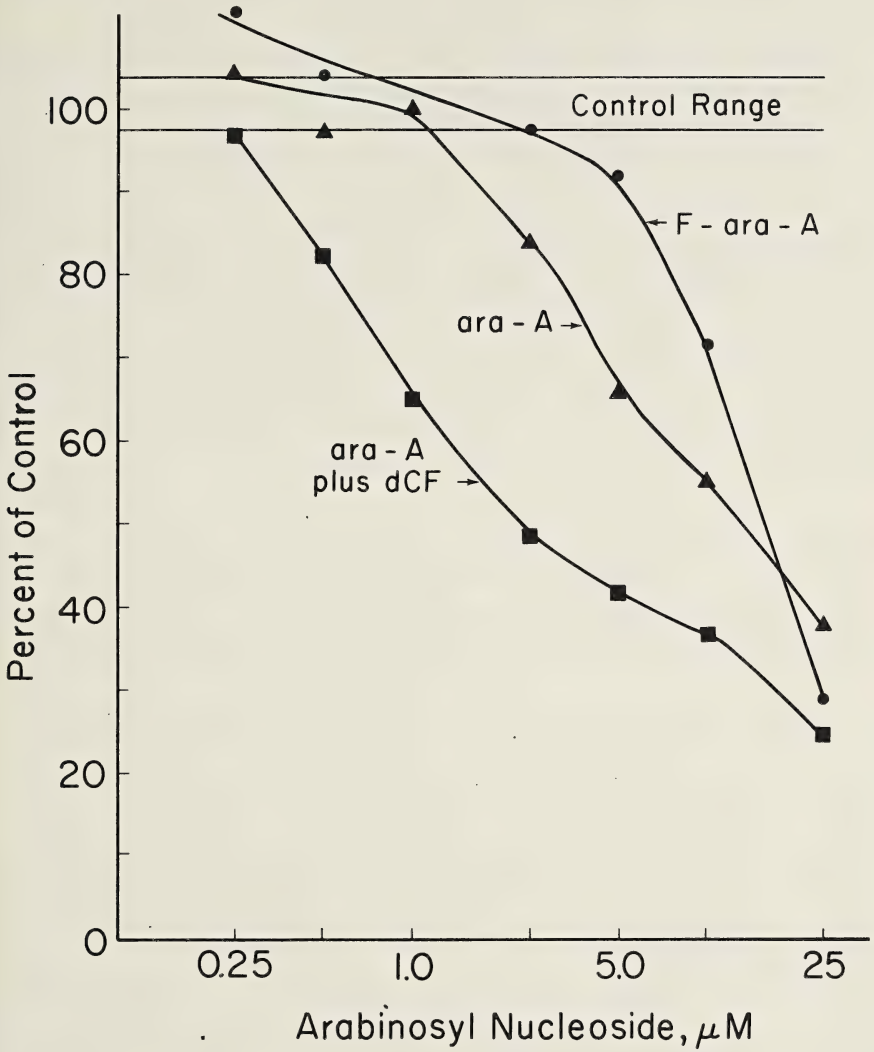

Figure 6. Effect of various concentrations of F-ara-A and of ara-A, either alone or in the presence of 1.0 μM dCF, on the incorporation of [^{3}H] thymidine by PHA-stimulated human lymphocytes.

incorporation by these cells except at higher (> 10 μM) concentrations. Preliminary analyses of cellular nucleotide pools after incubation with 100 μM ara-A plus dCF 100 μM F-ara-A indicate that similar concentrations of the respective triphosphates were present in the cells. The results suggest that F-ara-ATP is a less effective inhibitor of DNA synthesis than is ara-ATP in PHA-stimulated lymphocytes.

CONCLUSIONS

The metabolism and biological effects of F-ara-A have been compared with those of ara-A alone and in the presence of an inhibitor of adenosine deaminase in lymphoblastoid cells derived from a patient with acute lymphocytic leukemia and PHA-stimulated lymphocytes from a healthy individual. The sensitivity of ara-A to inactivation by deamination has been demonstrated in each system. Both cell types form the triphosphate of each nucleoside analogue and the DNA synthetic capacity of each cell type is sensitive to inhibition by both F-ara-A and ara-A. However, F-ara-A may be more inhibitory to the leukemia cell DNA synthesis than ara-A whereas the reverse is true in PHA-stimulated lymphocytes. Although results in these systems cannot now be directly compared or taken as indicators of drug efficacy in cancerous and normal cells, they do suggest that F-ara-A is a drug that may show some degree of therapeutic specificity for neoplastic cells.

ACKNOWLEDGEMENTS

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COMMENTS

Kouttab: In PHA-stimulated cells, is there a time-duration optimum, or peak, of stimulation of DNA synthesis?

Plunkett: Yes. In these experiments a peak of stimulation of DNA synthesis, more than 80 times that of controls, was observed 3 days after PHA addition.

Nagyvary: How does F-ara-A compare with ara-C with respect to development of analogue resistance?

Plunkett: Resistance to ara-C has been found to come by 2 main mechanisms: loss of the ability to phosphorylate the nucleoside and an increase in the amount of deaminase activity. By analogy, since F-ara-A is not susceptible to deamination, loss of the capacity to activate F-ara-A by phosphorylation might then be expected to be a major site, but not the sole site, at which resistance could develop. We have not yet isolated cells resistant to F-ara-A.

Nagyvary: Does incorporation of F-ara-A into DNA result in chain termination, or can the chain continue to be elongated?

Plunkett: Although nucleotides of both ara-A and ara-C have been shown to terminate DNA chains in cell-free systems, this does not appear to occur to a significant extent in intact cells. Although, by analogy, the action of F-ara-A would not be expected to differ greatly, as of this time we have no direct evidence bearing on this question.

O'Donovan: Ara-A is converted to ara-AMP by a *deoxy* kinase? Is there an adenosine kinase involved at all?

Plunkett: Several laboratories have reported that ara-A is not phosphorylated by adenosine kinase. A distinct deoxyadenosine kinase has been studied but its properties with respect to ara-A were not reported.

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EFFECT OF HEMIN ON CELL-FREE PROTEIN SYNTHESIS IN AVIAN RETICULOCYTES

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ABSTRACT

Hemin has been reported by several investigators to stimulate protein synthesis in rabbit reticulocytes, platelets and several types of mammalian tumor cells. This stimulation has been cited as an example of translational control of protein synthesis because the rabbit reticulocyte does not contain a nucleus. To determine if the effect of hemin on protein synthesis is specific to mammalian cells, we have studied its effect on protein synthesis in avian reticulocytes. Red blood cells obtained from anemic adult chickens and ducks were lysed in the presence of varying concentrations of hemin. Protein synthesis in these cell lysates were studied. No effect on protein synthesis was observed by the presence of any concentration of hemin employed. Neither was there any effect on protein synthesis obtained by the addition of hemin at the beginning of incubation to lysates prepared in the absence of hemin. Several reports indicated that in rabbit reticulocytes, hemin suppresses the formation of an inhibitor of protein synthesis, the hemin-controlled repressor (HCR). To determine what effect, if any, rabbit HCR had on avian globin synthesis, crude HCR was added at varying concentrations to a cell-free protein synthesizing system from adult chicken reticulocytes. The addition of rabbit HCR had no effect on chicken globin synthesis.

INTRODUCTION

Hemin has been shown to stimulate total protein synthesis (Lodish and Desalu, 1973) as well as globin synthesis (Bruns and London, 1965; Zucker and Schulman, 1968). This stimulation by hemin has been cited as an example of translational control of protein synthesis (London, *et al.,* 1976) because rabbit reticulocytes do not contain a nucleus. However, hemin has also been shown to stimulate protein synthesis in platelets from rabbits (Freedman and Karpatkin, 1973) and in several mammalian tumor cells including Krebs II ascites (Beuzard, *et al.,* 1973), Friend erythroleukemia (Dabney and Beaudet, 1977) and HeLa cells (Weber, *et al.,* 1975). Since the platelets and tumor cells contain a nucleus, the data implies that hemin may have a broad stimulatory effect on protein synthesis in mammalian cells or in all cells in general. The following study was undertaken to determine whether the stimulation of protein synthesis by hemin observed in mammalian cells is applicable to avian cells.

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MATERIALS AND METHODS

Preparation of Crude Lysates from Ducks, Chickens and Rabbits

The procedure of Brown, *et al.*, (1974) was used with the following alterations. Birds were made anemic by injecting 0.3 ml of 2.5% phenylhydrazine subcutaneously daily for 5 days. Blood was collected from the carotid artery of ducks and chickens on day 6. White blood cells were removed by aspiration after centrifuging whole blood at 3,000 x g for 5 min at 4 C. Hemin was added prior to cell lysis by using a 1 mM stock solution of hemin prepared as described by Weber, *et al.*, (1975). The supernatant obtained from centrifuging lysed cells at 17,000 x g for 20 min served as the crude lysate.

Preparation of Ribosomes and Crude Salt Wash from Chicken Reticulocytes

The procedure of Brown, *et al.*, (1974) was used with the following alterations. Crude lysate was layered over a 6 ml sucrose cushion (25 mM KCl, 5 mM $MgCl_2$, 50 mM TRIS--HCl pH 7.8, 1.5 M sucrose) and centrifuged at 105,000 x g for 8 hrs at 4 C. The middle third of the resulting supernatant provided the S-100 proteins. Crude polysomes were dissolved in Standard Sucrose Solution (0.25 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 3 mM $MgCl_2$) to yield a final concentration of 250 OD_{260}/ml. The solution was made 0.5 M KCl and gently stirred at 4 C for 30 min. Ribosomes were centrifuged at 105,000 x g for 2 hrs and the ribosomal pellet solubilized in Standard Sucrose Solution to yield 1 $OD_{260}/\mu l$. The supernatant, designated crude salt wash, was concentrated by dialysis against 50% glycerol, 50 mM Tris-HCl pH 7.8, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol.

Preparation of Crude and Partially Purified Hemin-Controlled Translational Repressor (HCR) from Rabbit Reticulocytes

Crude HCR was prepared as described by Kramer, *et al.*, (1973) and chromatographed on CM Sephadex C50 as described by Gross and Rabinovitz (1973). Ten μg of the partially purified HCR inhibited protein synthesis by 50% in the crude rabbit lysate incubated at 34 C for 20 min.

Assay for Protein Synthesis in Crude Lysates

Conditions for protein synthesis in crude rabbit lysates were those given by London, *et al.*, (1976) except that 0.04 mM concentration of the 20 amino acids minus leucine was used and leucine was used at 0.03 mM with 10 μCi ^{3}H-leucine. Incubation mixtures for protein synthesis with crude avian lysates contained the following in a final volume of 100 μl:50 μl crude lysate, 70 mM KCl, 3.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8, 1.56 mM ATP, 0.36 mM GTP, 15.36 mM creatine phosphate, 5 units creatine phosphokinase, 0.04 mM 20 amino acids minus leucine, 10 μCi ^{3}H-leucine. Incubations were done at 37 C and 20 μl

samples removed at indicated time intervals. Leucine incorporation into protein was assayed as the amount of radioactivity in hot trichloroacetic acid-precipitable materials.

Assay for Protein Synthesis in the Fractionated Cell-free System from Chicken Reticulocytes

Incubation mixtures contained the following in a total volume of 50 μl; 50 mM Tris-HCl pH 7.8, 70 mM KCl, 3.5 mM $MgCl_2$, 1.56 mM ATP, 0.36 mM GTP, 15 mM creatine phosphate, 3.8 units creatine phosphokinase, 0.04 mM 20 amino acids minus leucine, 5 μCi ^{3}H-leucine, 0.25 OD_{260} chicken liver tRNA, 0.5 mg S-100 total protein, 75 μg non-hemoglobin protein of crude ribosomal salt wash, 1 OD_{260} salt-washed ribosomes. Reaction mixtures were incubated at 29 C for 18 min or the indicated time intervals. Protein synthesis was assayed as the amount of radioactivity incorporated into hot trichloroacetic acid-precipitable materials.

RESULTS AND DISCUSSION

Effect of Hemin on Protein Synthesis in Avian Reticulocytes

The effect on protein synthesis in avian reticulocyte lysates prepared in the presence of varying concentrations of hemin is shown in Fig. 1B and C. The results show that in comparison with rabbit reticulocytes (Fig. 1A) hemin does not appear to stimulate protein synthesis in avian reticulocytes. Since the lack of a hemin effect in avian reticulocytes could have been due to the slight differences in reaction conditions required for protein synthesis in rabbit lysates, we measured the effect of hemin on protein synthesis in chicken lysates using conditions for the rabbit lysate. Protein synthesis was not stimulated.

It has been shown in lysates from rabbit reticulocytes that the addition of hemin after the start of incubation will stimulate protein synthesis (Adamson, *et al.*, 1969; London, *et al.*, 1976). This stimulation is directly proportional to the time when hemin is added with the greatest stimulation obtained by adding hemin at zero time. The results from adding 25 and 100 μM hemin to avian lysates prepared in the absence of hemin is shown in Fig. 2. The results show that addition of hemin in this manner has no significant effect on protein synthesis in these reticulocytes.

In rabbit reticulocytes it has been shown that the presence of hemin interferes with the formation of a protein called the hemin-controlled translational repressor (HCR) (Gross and Rabinovitz, 1972). The HCR from rabbit reticulocytes has been shown to inhibit initiation of protein synthesis (Balkow, *et al.*, 1973). Furthermore, HCR obtained from human erythrocytes has been shown to inhibit protein synthesis in rabbit lysates (Freedman, *et al.*, 1974). Since hemin does not appear to affect protein synthesis in avian lysates it became of interest to determine what effect mammalian HCR has on protein synthesis in chicken

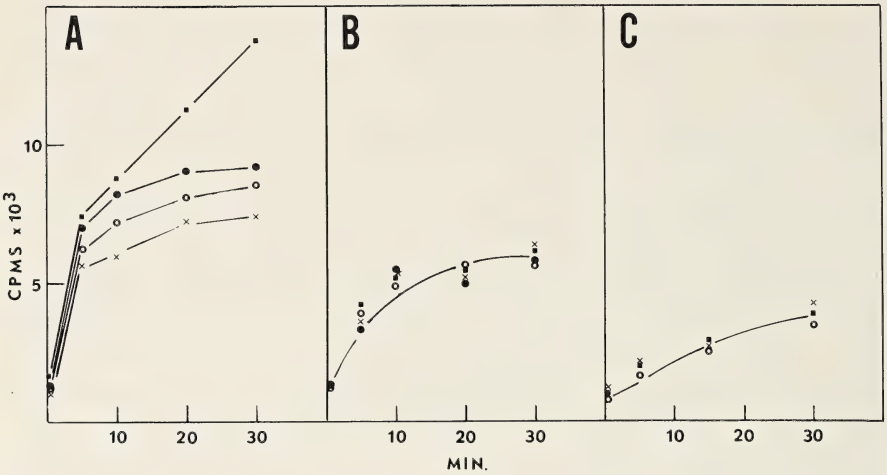

Figure 1. Effect of hemin on ^{3}H-leucine incorporation as a function of time in crude lysates of (A) rabbit, (B) chicken and (C) duck reticulocytes. Incubation conditions were as described under "Materials and Methods". o–o, no addition of hemin; ●–●, 15 μM; ■–■, 25 μM; x–x, 100 μM hemin.

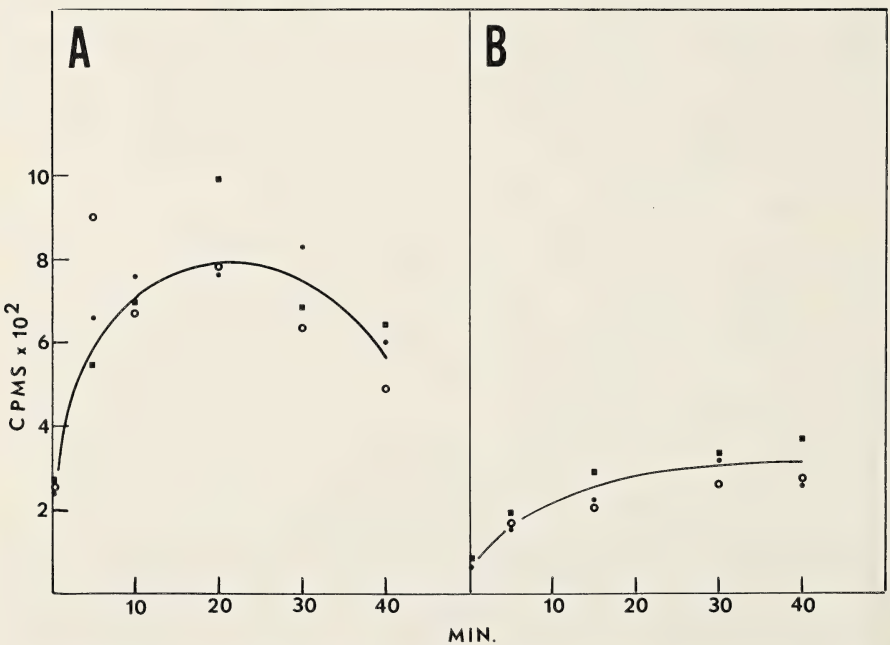

Figure 2. ^{3}H-leucine incorporation as a function of time with hemin added to crude lysates of (A) chicken and (B) duck reticulocytes prepared in the absence of hemin. Incubations were as described under "Materials and Methods". ●–●, no hemin added; ■–■, 25 μM hemin; o–o, 100 μM hemin. Each point represents the average of duplicates.

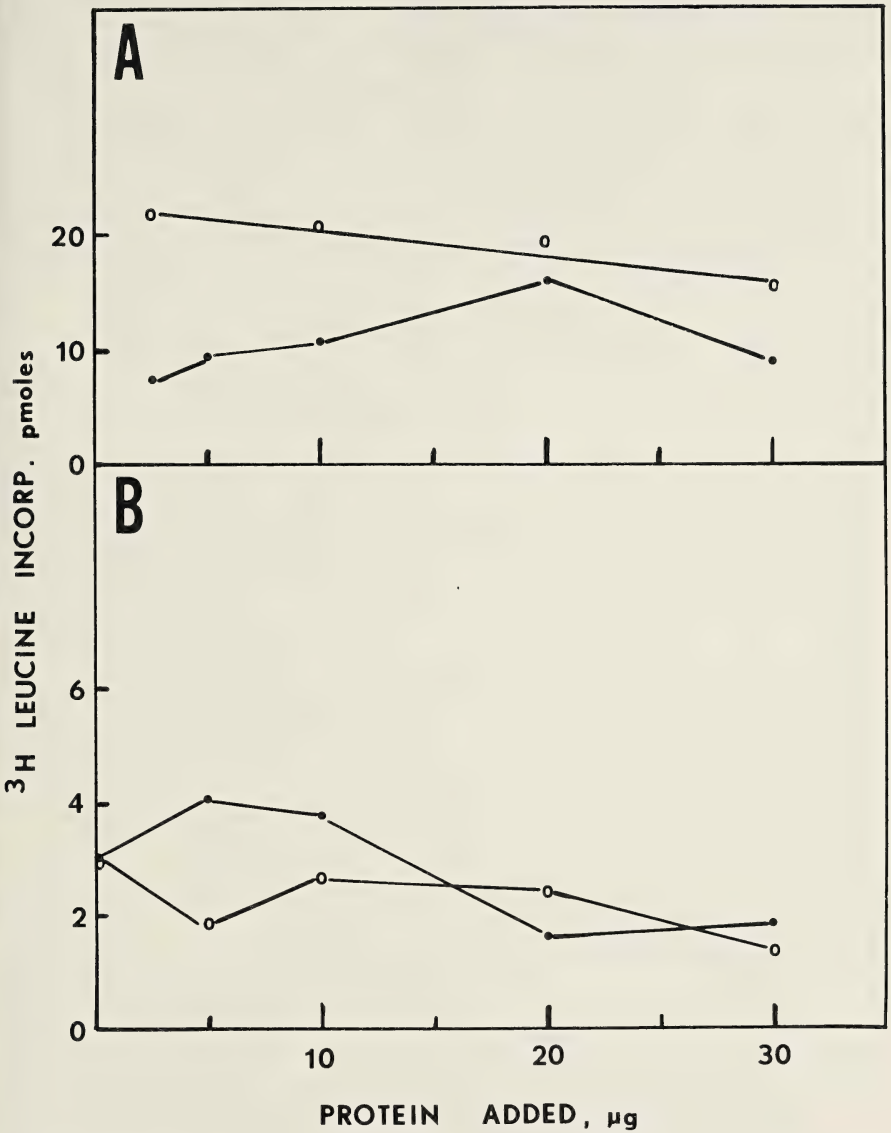

Figure 3. Effect of partially purified HCR from rabbit reticulocytes on protein synthesis in crude lysates of (A) chicken and (B) duck reticulocytes. Each point represents the average of duplicates and incubations were done as described under "Materials and Methods". o–o, partially purified rabbit HCR. •–•, protein obtained from rabbit reticulocytes lysates incubated in the presence of 25 μM hemin and chromatographed as described for the purification of HCR under "Materials and Methods". 10 μg of the partially purified HCR inhibited protein synthesis in rabbit lysate by 50% compared with the control material.

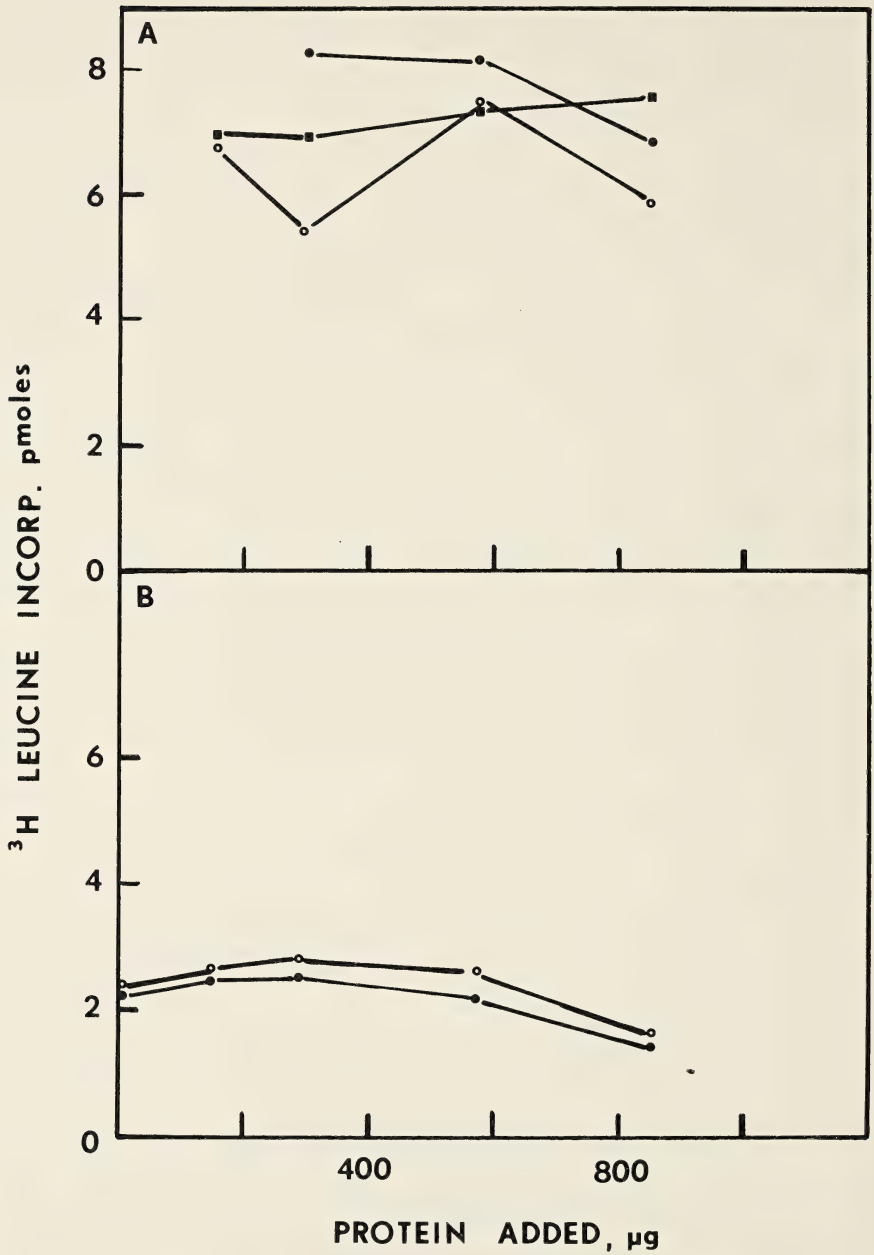

Figure 4. Effect of crude HCR from rabbit reticulocytes on protein synthesis in crude lysates of (A) chicken and (B) duck reticulocytes. Each point represents the average of duplicates. o–o, crude HCR; ●–●, rabbit reticulocyte protein incubated in the presence of 25 μM hemin as described under "Materials and Methods"; ■–■, BSA.

and duck reticulocytes. Partially purified HCR from rabbit reticulocytes was added at varying concentrations to avian lysates. The data (Fig. 3) indicate that partially purified HCR from rabbit does not affect protein synthesis in the avian systems.

To exclude the possibility that partial purification of rabbit HCR had affected its activity in avian lysates, crude HCR was added at varying concentrations. The results, (Fig. 4) which are similar to those obtained from the addition of partially purified HCR, showed that crude rabbit HCR had no detectable effect on avian protein synthesis. Bovine serum albumin also had no effect. However, it could be

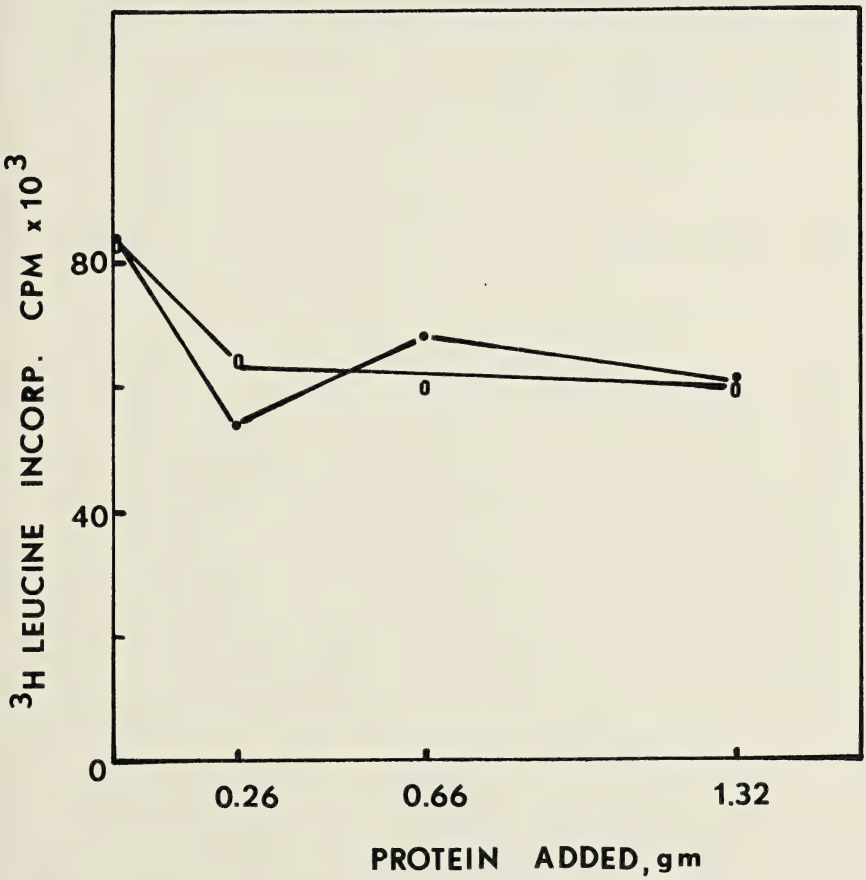

Figure 5. Effect of crude rabbit HCR on protein synthesis in a fractionated cell-free protein synthesizing system developed from chicken reticulocytes. Each point represents the average of duplicates. o–o, crude HCR; •–• control protein obtained as described in the Legend to Fig. 4.

argued that the crude lysates may not provide a system sensitive enough to detect low HCR activity. Therefore, the effect of rabbit HCR was measured in a fractionated cell-free protein synthesizing system developed from chicken reticulocytes. This system, described in Materials and Methods, employs S-100 protein, 0.5 M KCl ribosomal protein extract and the salt-washed ribosomes. The results from the addition of the crude rabbit HCR to this system are shown in Fig. 5. The data indicated that rabbit HCR does not affect globin synthesis in the fractionated cell-free system.

In summary, neither the presence of hemin nor mammalian HCR appear to affect protein synthesis in avian reticulocytes. Thus, the stimulation of protein synthesis by hemin may be a phenomenon restricted to mammalian cells and the mechanism of this stimulation may also be restricted to mammalian cells. In other words, the hemin controlled translational repressor may be operative only in cells capable of a response to hemin.

ACKNOWLEDGEMENTS

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SEARCH FOR TYPE C VIRUS-LIKE INFORMATION IN NORMAL, BENIGN AND MALIGNANT HUMAN PROSTATIC TISSUES

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ABSTRACT

A competition radioimmunoassay (RIA) was employed to examine the possible presence of type C virus-like antigen in human prostatic tissues. The assay was designed to detect the p30 interspecies antigen in murine leukemia virus (MuLV) preparations. In this assay human prostate tissue extracts were used in competition with the MuLV p30 interspecies antigen. The presence of MuLV p30-like antigen in human prostatic tissue was considered positive when the tissue displaced at least 30% of the ^{125}I-MuLV p30 interspecies antigen. The results were positive for 5 of 17 tissues from benign prostatic hyperplasia and 4 of 22 prostatic carcinoma. Negative results were obtained with extracts of 10 normal prostates. Two different tests using polyacrylamide gel electrophoresis and ^{3}H-casein coupled to Sepharose 4B demonstrated that enzymes such as proteases did not contribute to the positive results obtained. The results of these studies indicate that further investigations are justified to search for type C viruses or their components in human prostatic neoplasia.

INTRODUCTION

Virus particles resembling 2 major groups of viruses have been observed in prostate carcinoma: those belonging to the herpes-type viruses present in tumor cells obtained from prostatic carcinoma patients and in tissue culture cells (Tannenbaum and Lattimer, 1970; Weber, *et al.*, 1973; Brehmer, *et al.*, 1973; Centifanto, *et al.*, 1973; Sanford, *et al.*, 1975), and virus particles resembling type C viruses present in prostatic carcinoma (PCa) and benign prostatic hyperplasia

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(BPH) (Dmochowski, *et al.,* 1975; Dmochowski and Horoszewicz, 1975; Ohtsuki, *et al.,* 1976; Ohtsuki, *et al.,* 1977). Type C-like viruses have also been observed in mouse prostatic tissue (Ohtsuki, *et al.,* 1975). Seal, *et al.,* (1976) using extracts of BPH tissues only, showed by a competition radioimmunoassay (RIA) for the mammalian type C viral interspecies p30 antigen, that such extracts displace the p30 interspecies antigen. Mickey, *et al.,* (1977) reported the presence of a protein in BPH and PCa tissues which is antigenically similar to the p30 core protein of an oncogenic RNA virus; however, no mention was made of apparently normal prostatic tissue. The presence of murine leukemia virus (MuLV) p30 antigen in mouse prostate has also been reported (Kind, 1977).

In the present study, the competition RIA for type C virus interspecies p30 antigen was used to investigate the presence of MuLV p30-like antigens in normal, hyperplastic, and malignant human prostate tissues.

MATERIALS AND METHODS

Tissue Extracts

Prostate tissues were obtained frozen in dry ice from Dr. T.I. Malinin (University of Miami, School of Medicine, Department of Surgery, Miami, Florida) through the National Prostatic Cancer Project, or tissues were obtained immediately after surgery from the Department of Urology, M.D. Anderson Hospital and Tumor Institute, Houston, Texas. Tissues from PCa, BPH, and normal prostates were homogenized at 30% (w/v) in an omnimixer in extraction buffer composed of 0.01 M EDTA, 0.01 M Tris-HCl, 1% Triton in 0.1 M NaCl solution. The homogenates were then centrifuged at 60,000 x g for 30 min, and the supernatants passed through 0.45 μm Millipore filters. They were then stored at -70 C until needed.

Reagents

All reagents used in these studies were obtained from Dr. J. Gruber of the Office of Program Resources and Logistics of the National Cancer Institute.

These reagents included purified Rauscher leukemia virus p30 antigen, and goat antisera against Feline leukemia virus (FeLV), or FeLV p27 to detect the interspecies p30 antigen of MuLV, and goat antisera against MuLV p30 for comparison. Since this assay uses a double antibody system, a pig anti-goat IgG antiserum was also obtained.

Radioimmunoassay

Group specific antigens were labeled with ^{125}I to high specific activity by the Chloramine T method. This procedure was initially described by Greenwood and Hunter (1963), and has been used by others (Scolnick, *et al.,* 1972; Parks and Scolnick, 1972; Fowler, *et al.,* 1975). The radioimmune precipitation assay used in these studies has been described previously (Fowler, *et al.,* 1975). In

brief, 2-fold dilutions of prostatic tissue extracts in phosphate buffer containing 20% normal rabbit serum, pH 7.5, were incubated with anti-FeLV serum with antibody of a titer that precipitated 50% of the MuLV p30 interspecies antigen. After a 2 hr incubation at 37 C, ^{125}I-MuLV p30 antigen diluted to contain 10,000 cpm/100 μl was added to the mixture and incubated at 37 C for 1 hr and then overnight at 4 C. The following day a second serum with antibody (pig anti-goat IgG) directed against the first antibody (goat anti-FeLV p27) was added. The mixture was then incubated for 1 hr at 37 C and then for 2 hrs at 4 C. The reaction mixtures were centrifuged at 2500 rpm for 20 min and the supernatants counted in a gamma-spectrometer (Searle, Model 1197). In the present studies, prostate tissue extracts were used in competition with ^{125}I-MuLV p30 for reaction with anti-FeLV p27 antibody.

Protein Determination

Protein concentrations were determined according to the method of Lowry, *et al.*, (1951), with bovine serum albumin as a standard.

RESULTS

Antibody Titration

To determine the antibody titer that will precipitate 50% of the ^{125}I-MuLV p30 interspecies antigen, goat anti-FeLV serum at various dilutions was reacted with the interspecies antigen. The amount of MuLV p30 antigen iodinated was 14.5 μg. After the appropriate incubation, pig anti-goat serum was added; and after additional incubation as described for the RIA, the complexes were separated by centrifugation, and the supernatants counted for radioactivity. The antisera used and the results of titration experiment are presented in Table 1. In

TABLE 1

Titration of Anti-FeLV and Anti-FeLV p^{27} Sera with ^{125}I-MuLV p30 Antigen

Serum	Specificity	n[1]	Titer of Serum Precipitating 50% of ^{125}I-MuLV p30
AF 27	Goat anti-FeLV p27	3	0
2S-655[2]	Goat anti-FeLV p27	3	1:200
1S-132[2]	Goat anti-tween ether extracted FeLV	5	1:10,000

[1]Number of experiments

[2]Sera were obtained from the Office of Program Resources and Logistics, NCI.

several experiments only goat anti-tween ether extracted FeLV serum (IS-132) showed a high titer (1:10,000) for precipitation of the MuLV p30 interspecies antigen. Due to the consistency of results of titration experiments obtained with IS-132 reagent, this antiserum was used for the majority of these studies. The titer of antibody that precipitates 50% of the ^{125}I-MuLV p30 intraspecies antigen was found to be at a dilution of 1:100,000. These titers were then used to construct the standard RIA graphs for both the intraspecies and interspecies p30 antigens shown in Fig. 1. Thus the antisera used were capable of detecting the p30 antigen, and could be used to search for such information in human tissue.

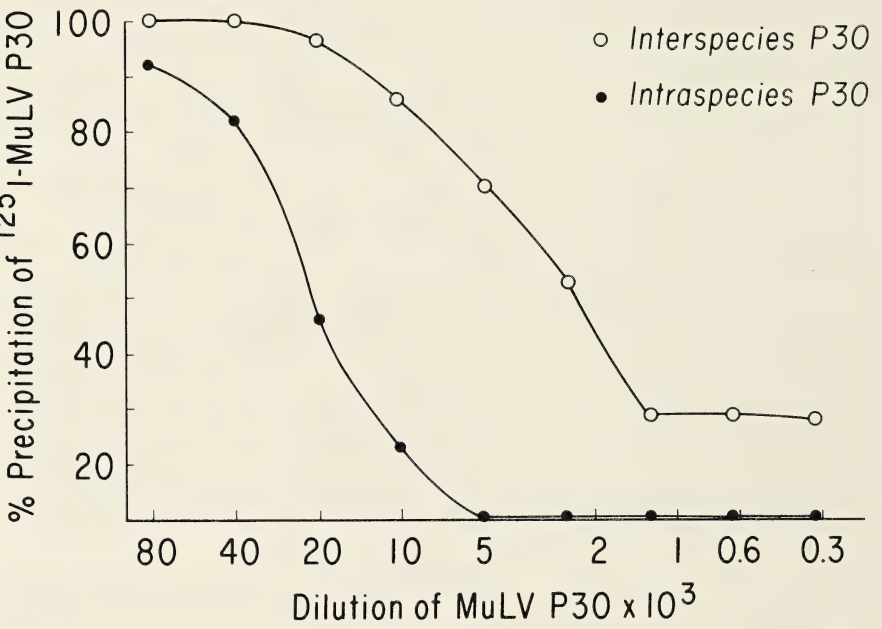

Figure 1. Radioimmunoassay showing the standard curves for the intraspecies, -●, and interspecies, -o, MuLV p30 antigen. The antisera used were goat anti-MuLV p30 ($1:10^5$ dilution) for the intraspecies antigen, and goat anti-FeLV (tween-ether extracted, $1:10^4$ dilution) - for the interspecies antigen. The amount of MuLV p30 protein iodinated was 14.5 μg.

Group Specific Antigens in Prostatic Tissues

Tissue extracts were competed with the ^{125}I-MuLV p30 interspecies antibody for reaction with the anti-FeLV antibody. The competition was considered positive when at least 30% of the ^{125}I-MuLV p30 interspecies antigen was displaced. The results were considered positive for 5 of 17 BPH, and 4 of 22 PCa which included a metastasis to the supraclavicular lymph node. Figure 2 is a graphic representation of the RIA slopes obtained for BPH and PCa tissue extracts.

Figure 2. Radioimmunoassay showing displacement of ^{125}I-MuLV p30 antigen by extracts from benign prostatic hyperplasia, –o, and prostatic carcinoma, –▲ Positive results (>30% displacement of antigen) were obtained when goat anti-FeLV antibody ($1:10^4$ dilution) was used to detect the interspecies of p30 antigen, –A. Negative results were obtained with goat anti-MuLV p30 antibody ($1:10^5$ dilution) which detects the intraspecies antigen, –B. The amount of MuLV p30 protein iodinated was 14.5 μg..

This figure shows that positive reactions were obtained in the interspecies p30 reaction but not the intraspecies p30 reaction. The extracts were positive at total protein concentrations of 0.063 ng (BPH), and 0.22 ng (PCa). To date, 10 normal prostate tissues were examined but no positive results were obtained with any of these tissue extracts. It should also be mentioned that positive tissue extracts were examined with an antibody specific for FeLV p27 obtained from Dr. A. Fowler (NCI, Bethesda, MD) and similar results were obtained as with the IS-132 antiserum.

Specificity of RIA Reactions

It became important to demonstrate that the positive reactions obtained were specific and not due to enzymes such as proteases. The observation (Fig. 2) that tissue extracts from BPH and PCa were positive with the interspecies p30 reaction and negative with the intraspecies p30 reaction indicates that these reactions are specific and that proteases were not involved in the reactions. That certain tissues can give positive reactions with both intraspecies and interspecies reactions is shown in Fig. 3. This figure shows the RIA slopes obtained with an extract from PCa tissue with sera against MuLV p30 and FeLV. Such reactions are considered nonspecific and therefore negative for MuLV p30-like protein. To confirm further the specificity of the positive results, ^{125}I-p30 antigen was

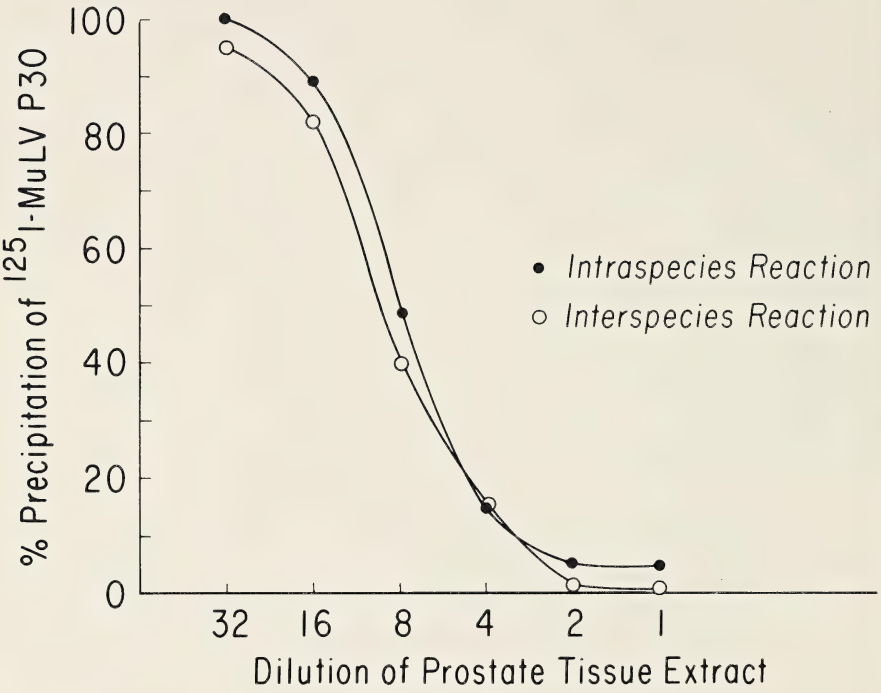

Figure 3. Radioimmunoassay of a human prostate cancer extract. The extract displaced the ^{125}I-MuLV p30 interspecies antigen, -o (using anti-FeLV serum) and the intraspecies p30 antigen, -• (using anti-MuLV p30 serum). Such tissue extracts are considered negative for MuLV p30-like antigen. The amount of MuLV p30 protein iodinated was 14.5 μg.

incubated with or without prostate tissue extracts. The concentrations of the reagents and incubation protocol were as described for the RIA. The mixtures were then applied to 7.0% polyacrylamide gels at pH 9.5. A total of 12 samples including 4 controls (^{125}I-MuLV p30 incubated without tissue extracts) were examined. The gels were sliced and counted in a gamma-spectrometer. The results are presented in Fig 4. Three peaks were observed, one of which is the major peak of p30 antigen. The other 2 minor peaks are probably breakdown products from the ^{125}I-MuLV p30 antigen which may have been formed during iodination or incubation. The presence of proteases in tissue extracts is expected to destroy the MuLV p30 antigen, and the radioactivity would be lost through the gels or would cleave the antigen into smaller fragments to produce a pattern different from that of the controls. Although Fig. 4 shows a slight shift in the peak of the p30 antigen, it is believed that such a shift is not large enough to indicate breakdown by proteases, and therefore again suggests that the results obtained were not due to the activity of proteases.

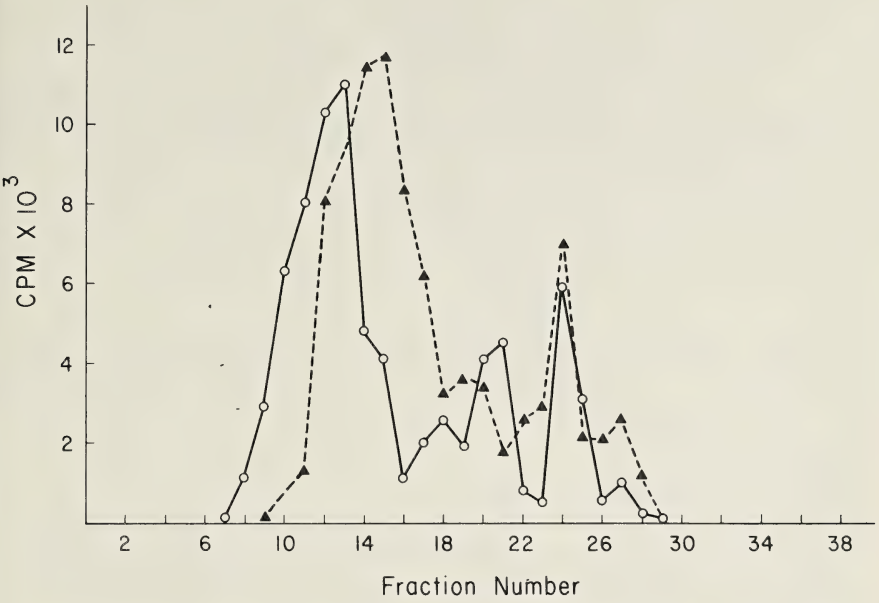

Figure 4. Polyacrylamide gel electrophoresis of ^{125}I-MuLV p30 antigen. The radioactivity patterns obtained from gel slices show no significant difference when the ^{125}I-MuLV p30 antigen was incubated with (-o) or without (-▲) extracts of prostatic carcinoma tissues. The incubation period and concentrations of MuLV p30 and prostatic tissue extracts were as used in the radioimmunoassay. The amount of MuLV p30 protein iodinated was 14.5 μg.

Still further confirmation of the specificity of the reactions was obtained by using Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) to which ^{3}H-casein has been coupled. A similar approach has been used by Hille and coworkers (1970). The presence of proteases in tissue extracts would cleave the protein and release the ^{3}H-casein into the supernatant. Therefore, a supernatant with high radioactive count would indicate a high protease activity. Prostate tissue extracts were incubated with ^{3}H-casein-Sepharose 4B. As control 0.25% trypsin was also incubated with the beads. Table 2 shows the results of this experiment. The range of spontaneous release of ^{3}H-casein was approximately 1000 CPM, therefore cpm above this number were considered positive for protease activity. Tissue extracts that were positive with intraspecies and interspecies p30 reactions also showed high protease activity as can be judged from the release of ^{3}H-casein. Thus, these tissue extracts showed as much enzyme activity as that obtained with trypsin. However, tissue extracts that were positive only in the interspecies reaction did not show higher release of ^{3}H-casein than obtained by spontaneous release. This was true for tissue extracts of BPH and PCa. The results indicated that extracts of normal tissues were negative for p30 interspecies antigen.

TABLE 2

Release of (^{3}H)-Casein by Proteases in Prostate Tissue Extracts

Tissue Extract	Release of (^{3}H)[1] CPM Mean and Range	RIA Reaction Intraspecies p30	Interspecies p30
Control	552 (296-875)		
0.25% Trypsin (50 μl)	3974 (2212-6097)		
PCa[2]	2038 (1045-4492)	+	+
PCa	718 (552-836)	-	+
BPH[3]	1465 (1003-1948)	+	+
BPH	482 (388-589)	-	+
Normal Prostate	1202 (1093-1346)	+	+

[1] Spontaneous release of (^{3}H).
[2] Prostatic carcinoma.
[3] Benign prostatic hyperplasia.

These results suggest that it may be possible to separate RIA reactions that are specific for p30 interspecies antigen from reactions that may be due to protease activity.

DISCUSSION

The present study using a competition RIA demonstrated the possible presence of antigens in human BPH and PCa tissues which displaced the interspecies p30 antigen of MuLV. This suggests that type C-like viral information may be present in these tissues. No such information has been detected in 10 tissues of normal prostates.

The presence of oncornavirus-like p30 antigen in human BPH tissues (Seal, *et al.*, 1976), and in BPH and PCa tissues (Mickey, *et al.*, 1977), as well as in apparently normal mouse prostate (Kind, 1977) has been suggested. Although the present studies confirm and extend the observations for human prostatic tissues, the percent of positive results obtained are much lower than reported by other studies. In this regard, it is suggested that great caution should be exercised in interpreting the RIA reactions. In the present studies many positive reactions were obtained; however, a critical evaluation of the results indicates that many of these reactions are nonspecific, as can be seen in Fig. 3, and are therefore negative. It is assumed that such nonspecific reactions are due to the presence of proteases.

Experiments with polyacrylamide gels and with ^{3}H-casein coupled to Sepharose 4B gave further evidence that the displacement of the p30 interspecies antigen appears to be specific and not due to protease activity. Nevertheless, it should be mentioned that the extracts used are crude tissue extracts and as such

may contain normal components which can displace the MuLV p30 interspecies antigen. Thus, confirmation by other similar studies or using different approaches would clarify further the question of the presence or absence of type C-like viral information in human prostatic tissues.

The results of these studies do not necessarily implicate type C-like viruses in prostatic neoplasia. They provide, however, another criterion for the possible presence of type C-like viruses in human prostatic tissue and justify further studies on the role of these viruses in prostatic neoplasia.

ACKNOWLEDGEMENTS

This work was supported in part by grant CA 15438-05 from the National Cancer Institute, USPHS.

COMMENTS

Brady: If it is a protease, would you expect the % displacement to be dependent on the time of incubation with the isotope?

Kouttab: If a protease is responsible for displacement of the p30 interspecies antigen, then it is very likely that displacement may be dependent on time of incubation. However, a protease would displace both the intraspecies and the interspecies p30. Since it was shown that some prostate tissue extracts displace only the interspecies p30 antigen, such reactions are considered specific and suggest the presence of MuLV p30-like antigen in human prostate tissue.

Brady: Have you done any experiments which serve as non-specific controls? For example, have you used *E. coli* phage proteins as control antigens?

Kouttab: No *E. coli* phage proteins were used as control antigens. However, 3 different approaches, namely the intraspecies p30 reaction, polyacrylamide gel electrophoresis, and ^{3}H-casein coupled to Sepharose 4B, were used to test the specificity of the results obtained. A tissue extract that displaced the interspecies p30 antigen, but also altered significantly the results of the controls for any of the 3 tests, was considered negative for the presence of MuLV p30-like antigen.

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EXPRESSION OF A RABBIT GLOBIN GENE-CONTAINING HYBRID PLASMID IN *ESCHERICHIA COLI*

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ABSTRACT

We have previously described the construction of a rabbit globin gene sequence-containing plasmid (Col E1 - RG_2) from Col E1 DNA and a rabbit globin mRNA/cDNA heteroduplex. Col E1 - RG_2 has now been transferred to the minicell-producing strain P 678-54 in order to examine gene expression by the plasmid. Minicells containing the hybrid plasmid were capable of synthesizing RNA, which yielded 4 high molecular weight bands on formamide acrylamide gels. These bands were of altered molecular weight when compared to species directed by the parent plasmid. Immunologically cross-reactive proteins were detected by reaction with goat anti-serum to rabbit hemoglobin among the products synthesized in minicells containing Col E1 - RG_2. The extent of immunoprecipitation was dependent upon the amount of Col E1 - RG_2 directed protein added to the reaction, but insensitive to the presence of Col E1 derived proteins. Thus, it appears that the globin gene introduced into the plasmid is expressed in minicells.

INTRODUCTION

We have previously described the construction of a hybrid Col E1 plasmid which contained a gene sequence for rabbit globin (Wood and Lee, 1976). The construction of the hybrid plasmid proceeded via a general format which has since become very familiar. Purified rabbit globin mRNA was used as the template for avian myeloblastosis virus reverse transcriptase and a cDNA copy of the mRNA was synthesized. Poly A "tails" were added to the 3' termini of the cDNA/mRNA by deoxynucleotidyl transferase. The mRNA/cDNA heteroduplex was then inserted by the action of *Escherichia coli* DNA polymerase I and T_4 DNA ligase into purified Col E1 DNA which had been converted to the linear form by digestion with the restriction endonuclease Eco R_I. Transformation of *E. coli* C600 r_k-m_k-with the hybrid plasmid DNA was shown to be an efficient and stable event. The plasmid DNA from 1 of the transformed clones, Col E1-RG_2 was shown to have a molecular weight of $4.40 \pm 0.04 \times 10^6$ daltons. This plasmid was shown by hybridization to [^{3}H]cDNA to contain a rabbit globin

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gene sequence. Having determined that the plasmid Col E1-RG_2 contained a globin gene sequence, we wished to examine the expression of this sequence. Accordingly, the plasmid Col E1-RG_2 was transferred into the minicell-producing strain P678-54. Col E1 or Col E1-derived plasmids which are present in the cytoplasm of P678-54 are segregated into the minicells (Roozen, *et al.*, 1971). Although minicells contain no host chromosomes, minicells which contain plasmids are capable of plasmid-directed RNA and protein synthesis (Roozen, *et al.*, 1971). This system was chosen to examine the expression of the hybrid plasmid since, in the absence of the host chromosome, one sees only plasmid-directed synthesis.

MATERIALS AND METHODS

Minicells containing either Col E1 or Col E1-RG_2 were purified by sucrose gradient density centrifugation essentially as described by Roozen, *et al.*, (1971). The resuspended minicells were incubated with [^{14}C] uridine or [^{14}C] amino acids as described. To study the nature of the [^{14}C] uridine labeled RNA synthesized, minicells were lysed with 1 mg/ml lysozyme, 0.1 mM/ml EDTA, pH 8.0, and 1% sarkosyl. The lysates were subjected to electrophoresis on 4% polyacrylamide-98% formamide gels (Pindar, *et al.*, 1974). Nonradioactive RNA was extracted as described by Roozen, *et al.*, (1971), except that the phenol was replaced by a phenol-chloroform-isoamyl alcohol (50:50:1) mixture. To examine protein synthesis, minicells were lysed as described by Meagher, *et al.*, (1977). Immunoprecipitation studies were performed using the buffer system of Capecchi, *et al.*, (1974). Carrier α and β globin (30 μg each) were present. The goat antiserum raised against rabbit hemoglobin used in this study was purchased from Cappel Laboratories, Cochranville, Pennsylvania.

RESULTS AND DISCUSSION

When the lysates from minicells incubated with [^{14}C] uridine were subjected to formamide polyacrylamide gel electrophoresis, it could be seen that both the parent plasmid, Col E1, and the hybrid plasmid Col E1-RG_2 directed the synthesis of 4 major high molecular weight species, as shown in Fig. 1. Several variable low molecular weight species, which probably represent initiation products, were also present. To confirm the molecular weights of these RNA species, nonradioactive RNA was extracted from minicells containing either Col E1 or Col E1-RG_2 and subjected to electrophoresis. The molecular weight of each species, indicated in the insert to Fig. 1, was consistent with that obtained from the radioactive precursor labeling studies. In both cases, all 4 RNA species derived from P678-54 (Col E1-RG_2) were of slightly higher molecular weight when compared to the corresponding species derived from P678-54 (Col E1). That all of these species were of high molecular weight is a somewhat surprising finding.

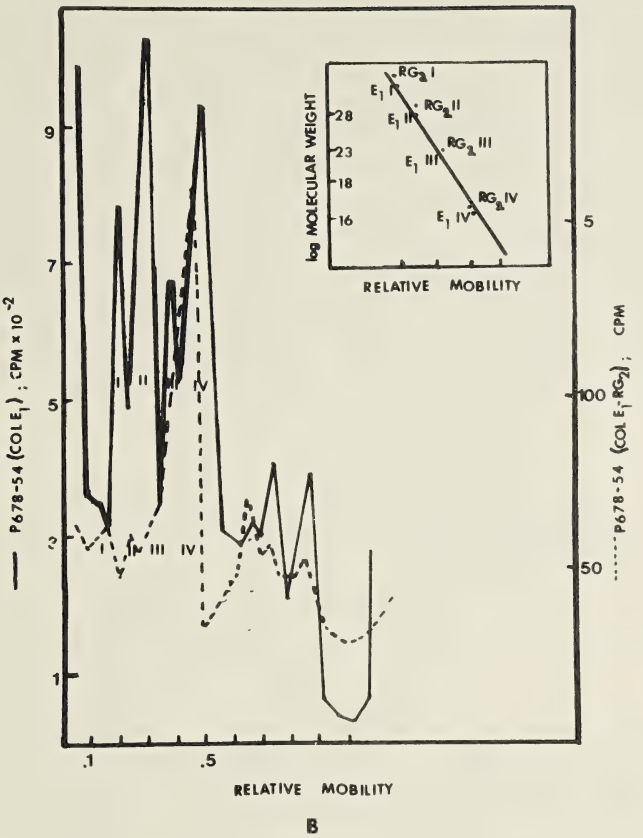

Figure 1. Electrophoresis on 4% polyacrylamide-98% formamide gels of RNA labeled in and extracted from purified minicells. RNA was extracted after 1 hr incubation with [^{14}C] uridine. The insert represents the plot of logarithm molecular weight *versus* mobility for nonradioactive RNA extracted from minicells.

The largest RNA species could account for the complete contour length of the plasmid DNA. It has been suggested by other workers (Grindley, *et al.*, 1977) that this is a general property of plasmid-directed RNA synthesis in minicells, and that it is due to faulty termination of transcription. This leads to the production of oversize RNA molecules, which may represent multiple rounds of transcription. This greatly complicated the interpretation of data obtained from RNA studies, and obscured identification of a discrete RNA species as a globin gene product. We, therefore, decided to examine expression of the rabbit globin gene sequence as a protein product.

The products of minicell protein synthesis were examined immunologically for a relationship to rabbit globin. Purified minicells were incubated with [^{14}C]

amino acids, collected by centrifugation and lysed. One μl samples were removed, and the total radioactivity incorporated determined. Samples of the lysate were utilized as antigen in the immunoprecipitation reaction. The radioactivity present in the precipitates was determined by liquid scintillation spectrometry. The results are shown in Table 1. Different batches of minicells synthesized protein at different levels; additionally, the parent strain always incorporated more label than the hybrid strain. The data were, therefore, normalized by dividing the counts/min precipitated by antibody by the total counts/min incorporated, to give the percent of incorporated radioactivity precipitated by antibody. Specific antibody precipitation occurred when the protein was derived from Col E1-RG_2 directed synthesis, but not when the protein was derived from Col E1 directed synthesis. Furthermore, the antibody precipitation was sensitive to the amount of Col E1-RG_2-derived protein present as antigen.

TABLE 1

Goat Anti-Rabbit Hemoglobin Precipitation of Lysates Derived from Minicells Containing Col E1 or Col E1-RG_2[1]

Strain	Antigen Prot. μg	Radioactivity Incorp. (CPM)[2]	Radioactivity ppt. by Ab (CPM)[3]	% Incorp. Radioactivity ppt. by Ab
P678-54	10	48,450	1,423	2.92
(Col E1)	30	138,200	2,095	1.51
	40	109,400	2,230	2.02
	50	205,500	2,897	1.40
P678-54	10	20,075	683	3.37
(Col E1-RG_2)	30	48,850	1,061	2.16
	40	49,050	4,412	9.12
	50	73,800	2,069	2.83
	100	116,325	789	0.64

[1] Immunoprecipitation was carried out as described by Capecchi, *et al.*, (1974) in the presence of 0.5% Triton X-100.

[2] A 1-μl sample was removed and assayed by liquid scintillation spectrometry in 2 ml of Bray's solution.

[3] The washed immunoprecipitate was solubilized in Protosol and assayed by liquid scintillation spectrometry in Aquasol as described in "Materials and Methods."

These data are displayed graphically in Fig. 2. It is clear that the degree of precipitation was dependent upon the amount of Col E1-RG_2 protein present. An antibody titration effect was seen. With increasing amounts of Col E1-RG_2 derived protein, a narrow zone of equivalence was reached. Increasing the protein concentration beyond that point caused a decrease in the percent of precipitable radioactivity.

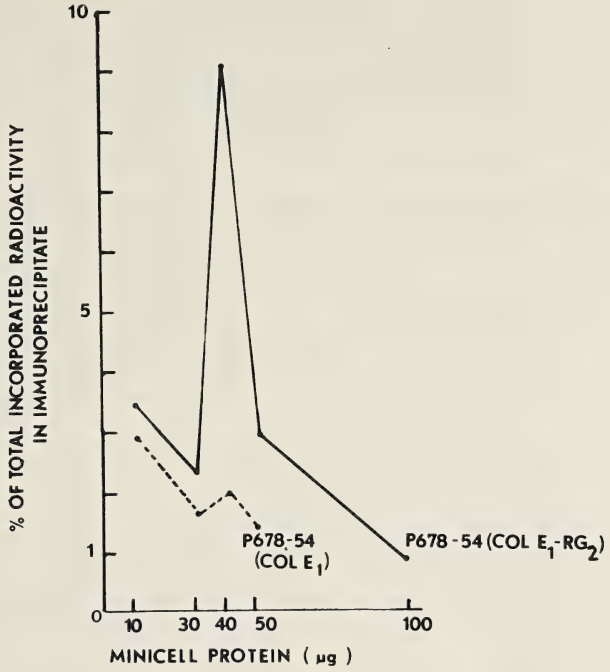

Figure 2. Assay of plasmid-directed protein synthesis by immunoprecipitation. Purified minicells containing either Col E1 or Col E1-RG_2 were incubated with [^{14}C] amino acids for 1 hr, lysed, and assayed for products immunologically related to rabbit globin by immunoprecipitation in the presence of 0.5% Triton X-100.

CONCLUSIONS

From these data we feel that the enzymatically synthesized globin gene placed in the *E. coli* minicells is expressed as a product which reacts with antibody raised against authentic rabbit hemoglobin. Work is in progress to characterize this product, and to determine the fidelity to rabbit globin.

ACKNOWLEDGEMENTS

This work was aided in part by the American Cancer Society Institutional Grant IN # 116 and Robert A. Welch Foundation Grant #AQ 541.

COMMENTS

Reudelhuber: Are the globin gene products detected directly in the SDS gels?

Wood: When the lysates from minicells containing Col E1-RG_2 were subjected to SDS gel electrophoresis, there were protein species of altered molecular weight present, as compared to lysates from minicells which contained Col E1. Identification of one of these bands as a globin gene product is being investigated by immunoreplication, but the autoradiographs have not been developed yet.

Zink: Have you tried inducing your minicell system with mitomycin C?

Wood: We have not tried inducing the minicell with mitomycin C, but we have tried inducing the C_{600} system. The results were somewhat equivocal, however, since the colicin E1 system does not induce as cleanly as does colicin E2 or colicin E3. There is so much chromosomal synthesis remaining that one gets a very high background.

Womack: What is the effect on plasmid copy number by this insertion?

Wood: Inserting the globin gene sequence decreases the copy number.

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POSSIBLE PLASMID-MEDIATED VIRULENCE IN *YERSINIA ENTEROCOLITICA*

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ABSTRACT

Strains of *Y. enterocolitica* obtained from an outbreak of human yersiniosis were examined for the production of heat stable toxin (ST), heat labile toxin (LT), and invasiveness (Sereny test). Analysis of the plasmid DNA complement of these strains by agarose gel electrophoresis showed that many of the strains contained a 35 megadalton (Mdal) plasmid and a 41 Mdal plasmid. The presence of the 41 Mdal plasmid was completely correlated with invasiveness (positive Sereny test). None of the strains produced LT. Although some of the strains produced ST, ST production was not correlated with a particular plasmid species. A non-invasive strain (negative Sereny test) originally isolated from a symptomatic child contains only the 35 Mdal plasmid, whereas serologically identical strains that are invasive contain both the 35 Mdal plasmid and the 41 Mdal plasmid or the 41 Mdal plasmid alone.

INTRODUCTION

Throughout the past decade, *Yersinia enterocolitica* has been the subject of intensive investigation as a cause of human disease. The organism has been etiologically incriminated in a variety of disease syndromes, including mesenteric lymphadenitis (Carlsson, *et al.*, 1964), acute terminal ileitis (Winblad, *1973),* and enterocolitis or gastroenteritis (Toma and Lafleur, 1974). In addition, biochemically atypical strains have been associated with eye, throat, wound and urinary tract infections (Bottone, *et al.*, 1974). Although these biochemically atypical strains resemble organisms isolated from vacuum-packaged meat (Hanna, *et al.*, 1976), the connection between human disease and strains of *Y. enterocolitica* isolated from foods and from the environment is circumstantial. To date, the only documented case of a foodborne outbreak of human yersiniosis, due to *Y. enterocolitica*, occurred in 1976 among school children in Oneida County, New York (Black, *et al.*, 1978). In this outbreak, *Y. enterocolitica* serotype 0:8

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was isolated from symptomatic persons and from a carton of chocolate milk obtained from the school cafeteria. Strains of *Y. enterocolitica* isolated during the epidemiological investigation of the Oneida County outbreak have been examined for the production of heat labile toxin (LT), heat stable toxin (ST), and the ability to invade epithelial cells (Sereny test) (Feeley, *et al., In Press*). Some of these strains produced ST and were able to invade the conjunctival epithelia of guinea pig eyes. None of the strains produced LT.

It is well known that strains of enterotoxigenic *Escherichia coli* produce ST and/or LT (Falkow, 1975). Genetic studies coupled with animal studies have shown that *E. coli* enterotoxin production is plasmid-mediated (Smith and Linggood, 1971; Gyles, *et al.,* 1974). Other plasmid-borne traits, such as hemolysin production and the production of certain cell surface antigens (i.e.,K88 or K99) are known to enhance the virulence of strains of *E. coli* (Smith and Linggood, 1971, 1972). In addition, virulent strains of *E. coli* often produce colicin V, and this property appears to be plasmid-mediated (Smith and Huggins, 1976). When the Col V plasmid (a plasmid which codes for colicin V) is eliminated from naturally occurring virulent strains of *E. coli* by a curing agent, the strains become significantly less virulent. Furthermore, *E. coli* K12 and other laboratory strains of *E. coli* become more virulent when they contain the Col V plasmid (Smith, 1974).

At the present time, plasmid-mediated enterotoxin production has not been demonstrated in members of the *Enterobacteriaceae* other than *E. coli*. Since plasmids are transferred between strains of *E. coli* and *Y. enterocolitica* (Cornelius, *et al.,* 1976), it is conceivable that strains of *Y. enterocolitica* could harbor enterotoxin plasmids, or other plasmids that mediate virulence. In this study, we report on the examination of the plasmid DNA content of strains of *Y. enterocolitica* isolated during the epidemiological investigation of the Oneida County outbreak.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Strains of *Y. enterocolitica* were isolated by the Special Pathogens Laboratory of the Center for Disease Control or by the New York State Health Laboratories. The characteristics of these strains are shown in Table 1.

The *E. coli* K12 strain C600 (F^-, *lacY1, thr-1, leu-6, thi-1, supE44, tonA21*) was employed as a standard host for each reference plasmid. The properties of the reference plasmids R6-5 (61 Mdal), RP4 (34 Mdal), Sa (23 Mdal), pSC101 (5.8 Mdal), and Col E1 (4.2 Mdal) have been described (Silver and Cohen, 1972; Datta, *et al.,* 1971; Watanabe, *et al.,* 1968; Cohen and Chang, 1977; Bazaral and Helinski, 1968). A trimer of plasmid pMB9 (10.5 Mdal) was obtained from R. Dixon, Baylor College of Medicine. The properties of plasmid pMB9 have been described (Rodriquez, *et al.,* 1976).

TABLE 1

Characteristics of Strains of *Y. enterocolitica*

Strain No.	Source	Serotype	LT Toxin	ST Toxin	Sereny Test
62	cow	4, 33	-	+	-
61	human	5	-	+	-
60	human	8	-	+	-
59	human	8	-	+	+
58	human	8	-	+	+
57	human	8	-	+	+
56	human	8	-	+	+
55	human	8	-	+	+
54	milk	8	-	+	+
53	human	5	-	-	-
63	water	6, 31	-	-	-

Isolation of Plasmid DNA

Plasmid DNA was isolated from cleared lysates of bacteria by the procedure described by Meyers, *et al.*, (1976). Purified plasmid DNA from *E. coli* K12, to be used as molecular weight standards, was isolated by the Triton-lysis method (Katz, *et al.*, 1973). Plasmid DNA obtained from the triton-lysis was further purified by dye-bouyant density-equilibrium centrifugation (Bazaral and Helinski, 1968).

Agarose Gel Electrophoresis of DNA

DNA purified from cleared lysates of strains of *Y. enterocolitica* was subjected to electrophoresis in 0.7% agarose (Miles Research Products) gels. Electrophoresis was carried out in a vertical slab apparatus [15.0 cm (L) x 9.6 cm (W) x 0.6 cm (T)] at 120V, 33mA for 3.5 hrs. The gels were stained for 30 min in electrophoresis buffer containing ethidium bromide (1.0 μg/ml). The stained gels were photographed on Plus-X pan film (Kodak) through a 23A and 15 orange filter combination. The electrophoretic migration of the DNA bands was measured from photographic prints.

Elimination of Plasmid DNA by Ethidium Bromide Treatment

Plasmid DNA was eliminated from strains of *Y. enterocolitica* by growing the cells in the presence of ethidium bromide (5 to 50 μg/ml). This was done according to the procedure described by Miller (1972), except that ethidium bromide was used in place of acridine orange.

Enterotoxin Assay

Strains of *Y. enterocolitica* were tested for the ability to produce ST and LT according to the procedure of Morris, *et al.*, (1976).

Sereny Test

The Sereny test was performed as described previously (Feeley, *et al., In Press*). The results were interpreted 48 hrs after inoculation into the guinea pig eye.

RESULTS AND DISCUSSION

Partially purified plasmid DNA's, obtained from each of 11 strains of *Y. enterocolitica*, were analyzed by agarose gel electrophoresis. In this analytical system, the migration of plasmid DNA (superhelical, covalently closed circular DNA) is inversely related to the molecular weight of the plasmid DNA. Fragments of chromosomal DNA (linear DNA) migrate as a diffuse "smear" beginning in the region of the gel where 12 Mdal plasmid DNA would appear, and extending to the bottom of the gel. Although the chromosomal DNA obscures a portion of the gel, this technique has proved to be a rapid means of determining whether or not a bacterium contains plasmid DNA. The technique has the added advantage of facilitating the estimation of the molecular weight of the plasmid DNA.

Figure 1 shows the results of such an analysis of 11 strains of *Y. enterocolitica*. The serotype 0:8 strains (slots c through h and slot j) were epidemiologically identified as the causative organisms. Each of the serotype 0:8 strains contains a 41 Mdal plasmid or a 35 Mdal plasmid or both. The strains of *Y. enterocolitica* isolated from cattle, water, or asymptomatic humans (slots a, b, k, and 1) did not contain plasmids of this molecular weight class, 1 strain, TAMU 61, contained a 47 Mdal plasmid and a 3.1 Mdal plasmid.

Table 1 lists the results of Sereny tests and ST tests on each of the strains of *Y. enterocolitica*. Only strains that contain the 41 Mdal plasmid are Sereny test positive (invasive). There is no obvious association between plasmid DNA content and ST production.

In order to investigate the association of the 41 Mdal plasmid more fully, we attempted to eliminate the 41 Mdal plasmid from strain TAMU 54. We were able to eliminate this plasmid by growing the cells in the presence of ethidium bromide at 35 C. Several strains which were "cured" of the 41 Mdal plasmid (Fig. 2)

Figure 1 (See following page).

Figure 1. Agarose gel electrophoresis of ethanol-precipitated DNA isolated from cleared lysates of strains of *Y. enterocolitica*. (a) Strain 62, 18 μl of ethanol-precipitated DNA. (b) Strain 61, 18 μl of ethanol-precipitated DNA. (c) Strain 60, 12 μl of ethanol-precipitated DNA. (d) Strain 59, 12 μl of ethanol-precipitated DNA. (e) Strain 58, 12 μl of ethanol-precipitated DNA. (f) Strain 57, 12 μl of ethanol-precipitated DNA. (g) Strain 56, 12 μl ethanol-precipitated DNA. (h) Strain 55, 12 μl of ethanol-precipitated DNA. (i) Purified standard plasmid DNA (10 μl): upper band, R6-5; second band, RP4; third band, Sa; fourth band, pMB9-trimer; fifth band, pSC101; sixth band, Col E1. (j) Strain 54, 12 μl of ethanol-precipitated DNA. (k) Strain 53, 18 μl of ethanol-precipitated DNA. (1) Strain 63, 18 μl of ethanol-precipitated DNA.

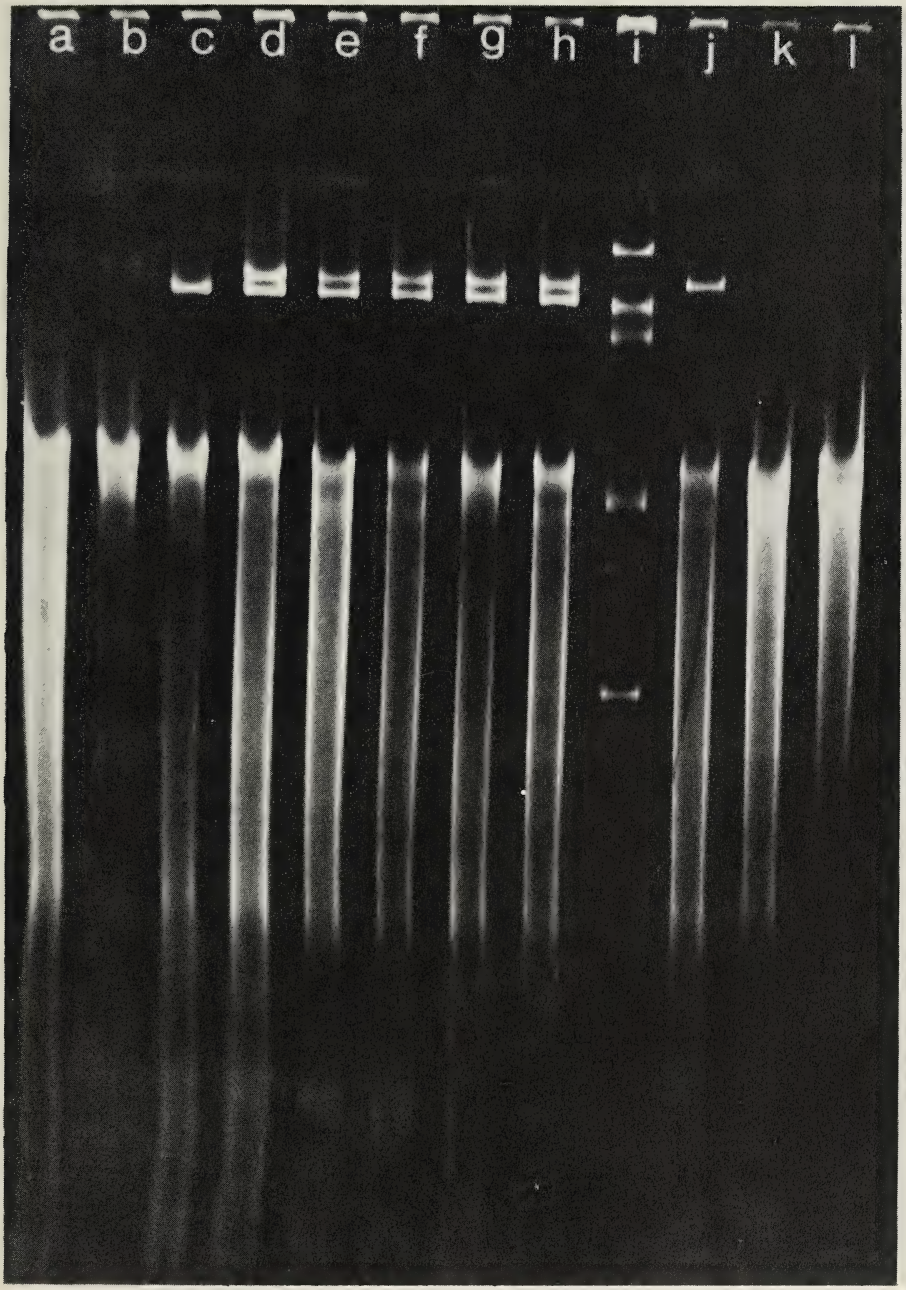

Figure 1. (Described on preceding page).

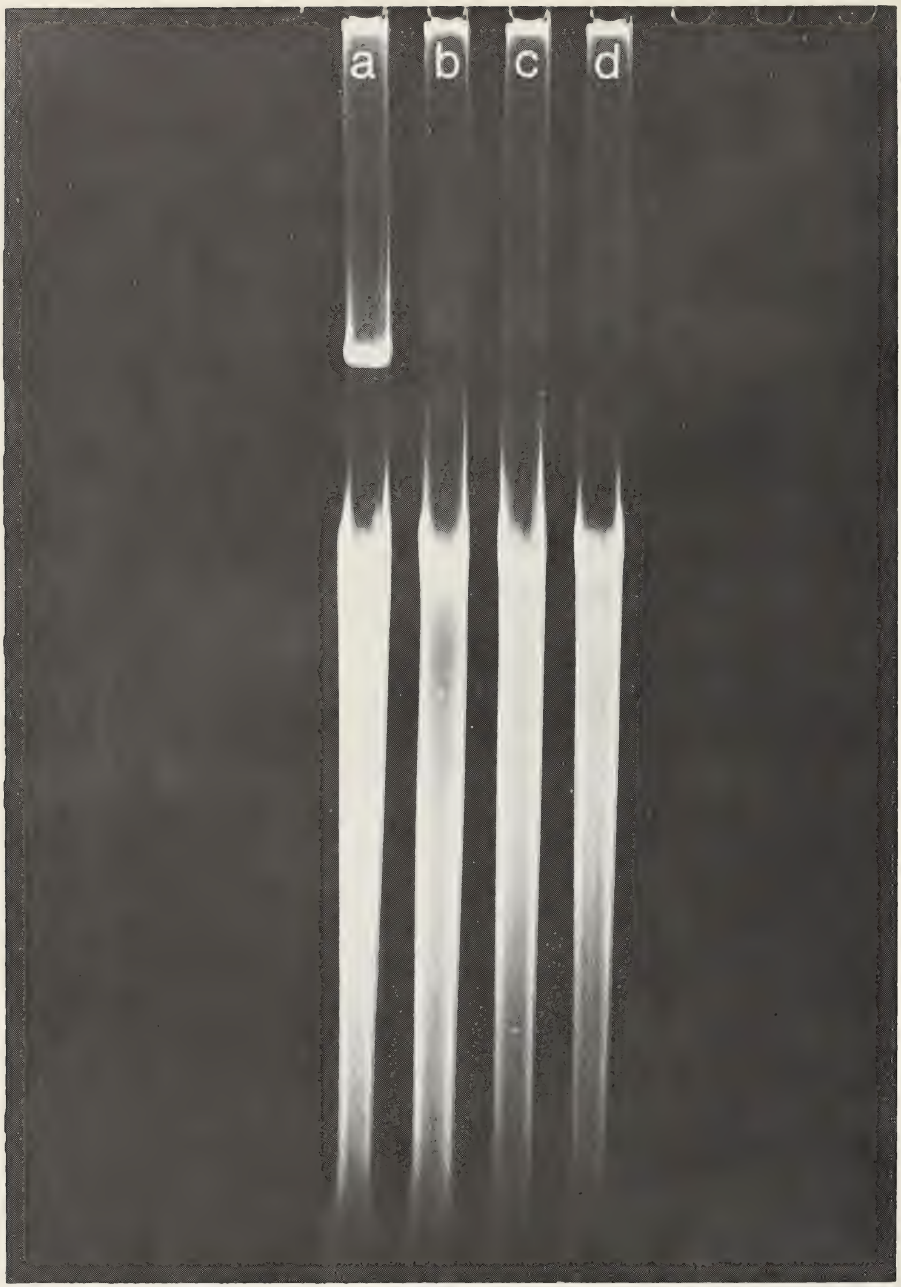

Figure 2. Agarose gel electrophoresis of ethanol-precipitated DNA isolated from strain 54 and strains derived by the ethidium bromide treatment of strain 54. The ethidium bromide-treated strains lack the 41 Mdal plasmid. (a) Strain 54, 25 μl of ethanol-precipitated DNA. (b) Strain 75, 25 μl of ethanol-precipitated DNA. (c) Strain 75-1, 25 μl of ethanol-precipitated DNA. (d) Strain 75-2, 25 μl of ethanol-precipitated DNA.

(TAMU 75, 75-1, 75-2) were tested for ST production and Sereny reaction. The plasmid-less strains were ST negative and Sereny test negative.

Although we have only examined one strain in detail, these data suggest that ST production and invasiveness (Sereny positive) in *Y. enterocolitica* could be plasmid-mediated. It is essential that other strains be examined in order to establish the genetic basis for these phenomena associated with pathogenicity.

CONCLUSIONS

Pathogenic strains of *Y. enterocolitica* are capable of producing ST and are able to invade the epithelial cells of the guinea pig eye. The data presented here suggest that these phenomena are plasmid-mediated. Studies of this type should be applied to other strains of *Y. enterocolitica* and to other members of the *Enterobacteriaceae*. It is probable that plasmid-mediated virulence is common in other members of the *Enterobacteriaceae*. This possibility should be investigated in any outbreak of enteric disease.

ACKNOWLEDGEMENTS

D.L. Zink acknowledges a Travel Grant from the Southern Regional Education Board, Atlanta, GA.

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COMMENTS

Garrard: Do you take any of the physical containment precautions recommended by NIH?

Zink: We have a self imposed standard which approximates P2 containment. The NIH guidelines do not address the problems associated with working with naturally occurring plasmids that mediate the virulence of their bacterial host.

Nairn: The NIH guidelines do not apply to this work.

Zink: That's correct, the NIH guidelines apply only to research involving *in vitro* recombinant DNA.

Mattox: Have you examined *Y. pestis* for this plasmid?

Zink: No. The organism is simply too hazardous for us to attempt to work on it. We would want better biohazard containment facilities.

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ABSTRACTS
OF
9TH ANNUAL
GULF COAST MOLECULAR BIOLOGY CONFERENCE

Sheraton-Marina Inn
Corpus Christi
January 27 - 28 - 29, 1978

Abstracts: 9th Annual Meeting of the Gulf Coast Molecular Biology Conference, Sheraton-Marina Inn, Corpus Christi, Texas, January 27 - 28 - 29, 1978

COLLAGEN BIOSYNTHESIS BY HAMSTER LUNG CELLS.

Michael A. Haralson and William M. Mitchell, *Department of Pathology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232.*

The HT1 clone of cultured Chinese hamster lung (CHL) cells devotes a major portion of its biosynthetic capacity to the production of collagen. Cell-free extracts from CHL cells incorporate proline into newly synthesized material with a significant fraction of the product being digestable by purified bacterial collagenase. Analysis of the *in vitro* products indicates 3 major forms (MW's = 165K, 100K & 65K) of collagenase-sensitive material with each having a corresponding collagenous product secreted by the cells in culture. In the presence of the postribosomal supernatant fraction of wheat germ lysates, CHL polysomes direct the synthesis of a single molecular weight (170K) collagenase-digestable species which represents 20 to 30% of the total cell-free synthesized product. This material corresponds in size to the procollagen α chain suggesting that this entity is the initially synthesized gene product. These results demonstrate that CHL cells constitute the first practical model system, not only for the isolation of procollagen α chain mRNA, but also for dissection of the elements involved in the regulation of procollagen biosynthesis in the mammalian cell. (This research is supported by NIH Grant No. AM 18222).

COMMENTS

Garrard: In your identification of products you use 2 probes, proline incorporation and collagenase sensitivity. Have you considered using an immunological probe as an identification technique such as collagen antibodies?

Haralson: Yes, we have considered the use of such probes and are currently in the process of obtaining both collagen and procollagen isotype specific antibodies from Dr. George Martin at N.I.H.. We hope to use these antibodies to determine if CHL collagen is antigenically related to any of the known collagens. As yet we have not raised antibodies against the collagen(s) produced by CHL cells in culture but are considering such work. It should be pointed out that the production of collagen antibodies is a difficult procedure because the molecule(s) is a poor antigen; hence the immunological characterization of cell-free synthesized collagenous proteins is a more formidable task, relative to other cell-free synthesized proteins for which antibodies are readily available which probably explains why no one has used immunologic data to demonstrate the cell-free synthesis of collagenous proteins.

Henderson: Have you done any experiments which address the possibility that there is an inhibitor in the wheat germ system which selectively prevents the initiation of the 67,000 and 100,000 molecular weight product? For example, will the S-100 from the CHL cells cleave the single molecular weight cell free synthesized product produced in the wheat germ polysome system?

Haralson: Yes, the S-100 from CHL cells will partially degrade the 170,000 molecular weight product to molecules that correspond in size to the 67K and 100K products synthesized in the S30 system, an observation that is consistent with the

hypothesis that these products arise via proteolytic processing from initially synthesized procollagen α chains. Also, I failed to point out that the amount of collagenase-sensitive product produced in the 2 systems is quantitatively the same – i.e., 20 to 30% of the total product – which further supports the contention. These observations do not eliminate, however, the possibility that an inhibitor that prevents translation of CHL mRNA specifying the synthesis of collagenase sensitive proteins of the same size is present in the wheat germ S100. Furthermore, we cannot eliminate the possibility that a positive translational element required for the synthesis of the 67K and 100K products, similar to a myosin mRNA specific IF-MP or such as specific isoaccepting tRNA species, may be absent in the wheat germ S-100.

Lovelady: Does the 8-amino propiononitrile affect the cell-free system, or only the cells in culture?

Haralson: I don't know because I have never examined its effects in the cell-free system.

Vickery: Have you compared the characteristics of a cell-free system derived from early passage CHL cells with one derived from late passage cells?

Haralson: Let me preface my response by stating that to my knowledge Chinese hamster lung cells do not age in culture. Therefore, the capability to synthesize collagen of cell-free extracts prepared from current cultures of CHL cells in this laboratory appears to be the same as it was approximately a year ago, and during this time the cells have been passaged approximately 100 times which represents ~ 500 doublings (generations). Every 5 months we routinely do a karotype on the cells, and the line has maintained a modal chromosome # of 22. The CHL line (HTI) used in these studies was obtained from Don Roufa and represents a subclone of the V79 CHL line originally established by Ford and Yerganian, 1958 (*J. Natl. Cancer Inst.*, 21:393). In contrast to other cultured lung cells, particularly WI-38 fibroblasts, in which collagen production decreases as the cells age and lose division capacity, the fact that CHL cells do not age and hence maintain their ability to synthesize collagen constitutes a further advantage offered by this cell line for studying the biosynthesis of collagen in mammalian cells.

In the light of the interesting paper Zink, *et al.*, elsewhere in these proceedings, perhaps it is worth mentioning at this time that suggestive evidence exists to imply that genetic recombinant "experiments" involving *E. coli* and human DNA have already and are presently occurring. One laboratory has reported (Cohen, H. and S. Strampp, 1976, *Proc. Soc. Exptl. Biol. & Med.*, 152:408) the isolation from the urine of a patient with colonic carcinoma of a "bacterial" organism which secretes into its growth medium a substance similar to HCG (human chorionic gonadotropin) a 2 subunit polypeptide hormone synthesized by the placenta during the 1st trimester of pregnancy and thought to play some role in the maintenance of fetal integrity. The production of such a mammalian specific protein in culture by an organism tentatively classified by the investigators as *E. coli* suggests that a "shotgun-type" recombinant situation occurred between the normal intestinal flora and human genes (DNA) liberated during colonic cell lysis as a result of the carcinoma. Furthermore, the authors allude to the observation that production of HCG by this organism is "unpredictable" suggesting that the gene insertion or transformation element is unstable; this might explain why "*E. coli*-like" organisms carrying other human genes have not been detected. The report does, however, support the hypothesis shared

by several investigators that interphyla genetic recombination occurs spontaneously in nature and that the "construction" of such organisms in the laboratory would be nothing new. The data also imply that the potential "danger" of *in vitro* DNA recombinant experiments is not the creation of bacteria carrying human genes but rather the construction of such an organism in which the new DNA is integrated in a stable manner.

O'Donovan: I am quite skeptical of the work of Cohen and Strampp even though I believe that many kinds of recombinational events occur in nature. Cohen and Strampp received two cultures from the Livingstons (V. Livingston and A.F. Livingston, 1974, *Trans. N.Y. Acad. Sci.,* 36:569) and identified them as *E. coli*. However the organisms sent by the Livingstons in two cultures were both gram positive cocci.

PROBING THE TOPOGRAPHY OF HISTONE H1 AT THE NUCLEOSOMAL SURFACE.

Parthenios Boulikas and William T. Garrard, *Department of Biochemistry, The University of Texas Health Science Center at Dallas 75235.*

Histone H1 species have been implicated to play roles in gene inactivation, nucleosome spacing, and higher order coiling of chromatin fibers. In the present study, protein cross-linking reagents have been utilized in order to understand how histone H1 interacts with nucleosomes at the molecular level. The chemical probes used include dimethylsuberimidate and a carbodiimide. Two-dimensional gel electrophoresis has been employed to map the distributions of DNA fragments or cross-linked histone products among mono- and polynucleosomes. It is concluded that mononucleosomes which contain histone H1 species have *one* copy of this protein and 8 other histone molecules. It is concluded further that histone H1 electrostatically interacts with specific histones at the nucleosomal surface by (COO^-) (NH_3^+) contacts. (This research was supported by NIH and the National Foundation - March of Dimes.

COMMENTS

Belew: Have you used cleavable cross-linkers?

Boulikas: We have been using methyl-4-mercaptobutyrimidate (MMB) and dithiobis-succinimidyl propionate (DTSP); both of these reagents have a cleavable S-S bridge (the former after H_2O_2 oxidation following monofunctional attachment to ϵ-NH_2 groups). For limit crosslinking, dissociation of histones H1, H2A and H2B occurs after MMB treatment prior to oxidation, possibly due to the addition of negative S^- charges to the histones, although the amidination reaction itself retains the positive charge of the site attacked (the dimer S-S form of MMB is unstable). Note that the Arg-rich histones H3 and H4 do not dissociate because a great deal of their salt linkages with DNA is contributed by Arg residues which are not attacked by the reagent.

DTSP, on the other hand, attacks ϵ-NH_2 groups and causes dissociation of H1. In solution, association to H1-H1 dimers occurs due to the loss of ϵ-NH_2 positive charges induced by the reagent itself. However, both reagents gave valuable information on histone dimer composition in experiments when a low degree of modification was used.

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COVER PHOTO

A: Diagrammatic representation of DNA.
B: Commonly used diagram for DNA.

(Cover Photo drawn by Dr. E. N. Drake, II,
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